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Fish Protein Hydrolysates: Production, Biochemical, and Functional Properties

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ABSTRACT: Considerable amounts of fish processing byproducts are discarded each year. By developing enzyme technologies for protein recovery and modification, production of a broad spectrum of food ingredients and industrial products may be possible. Hydrolyzed vegetable and milk proteins are widely used food ingredients. There are few hydrolyzed fish protein foods with the exception of East Asian condiments and sauces. This review describes various manufacturing techniques for fish protein hydrolysates using acid, base, endogenous enzymes, and added bacterial or digestive proteases. The chemical and biochemical characteristics of hydrolyzed fish proteins are discussed. In addition, functional properties of fish protein hydrolysates are described, including solubility, water-holding capacity, emulsification, and foam-forming ability. Possible applications of fish protein hydrolysates in food systems are provided, and comparison with other food protein hydrolysates where pertinent.

KEY WORDS: fish protein hydrolysates, fish protein, functional properties, chemical hydrolysis of fish protein, enzymatic hydrolysis of fish protein, protein functionality, fish byproducts.

I. INTRODUCTION

At this time there are huge amounts of protein-rich byproduct materials from seafood processing plants discarded without any attempt of recovery. At the same time many processors are no longer allowed to discard their offal directly into the ocean, resulting in a very high cost of refining the material before it is discarded. To meet the need of the seafood processing industry, an alternative to discarding these byproducts should be developed. Recovery and alteration of the fish muscle proteins present in the byproduct material and using these as functional ingredients in food systems is a very exciting and promising alternative. However, for the industry to develop processes for byproduct recovery and utilization it has to be more economically feasible than discarding the byproducts.

Every year over 91 million tons of fish are harvested, of which 29.5% is transformed into fishmeal.[1,2] Possibly more than 50% of the remaining fish tissue is considered to be processing waste and not used as food.[3] With a dramatically increasing world population and a world catch of fish presently on the verge of exceeding the estimated sustainable limits of the suggested 100 million tons/year, there is obviously an increased need to utilize our sea resources with more intelligence and foresight. By applying enzyme technology for protein recovery in fish processing, it may be possible to produce a broad spectrum of

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food ingredients or industrial products for a wide range of applications. This would utilize both fishery byproducts or secondary raw materials and, in addition, underutilized species that would otherwise be discarded.

Enzymatic modification of proteins using selected proteolytic enzyme preparations to cleave specific peptide bonds is widely used in the food industry.[4] Hydrolysis of food proteins has a long history, mainly for vegetable and milk proteins; these proteins are widely used in the food industry. Most work on the hydrolysis of fish proteins was conducted in the 1960s. Some fish protein hydrolysate (FPH) preparations at that time were quite successful.[5] During the 1960s, research was directed to the production of cheap nutritious protein sources for rapidly growing developing countries, or toward animal feed production, primarily through production of fish protein concentrates (FPC). Little work has been done recently on FPH, but some research has been directed into the potential of using powdered hydrolysates in food formulations. Many studies have resulted in fish protein hydrolysates with excellent functional properties. However, taste defects, specifically bitterness, and process economics are still major limiting factors for FPH applications.

This review gives an overview of the different techniques for production of fish protein hydrolysates, past and present research on their properties, and various methods to study the extent of hydrolysis and product functionality.

II. THE BIOCHEMICAL CHARACTERISTICS OF FISH MUSCLE PROTEIN

In foods, a protein is traditionally categorized as a fibrous or globular protein based on its tertiary conformation. Each type of food protein has a unique molecular structure that determines its functional properties, in addition to a range of environmental conditions over which it exhibits such properties.[6] These factors and their effect on functionality are discussed in more detail later in this review.

The functional and structural properties of food proteins thus vary tremendously, and to fully understand the process of protein hydrolysis it is crucial to have a good understanding of the nature of the protein substrate and the hydrolyzing agent. During protein hydrolysate manufacture, the protein substrate is hydrolyzed by either a proteolytic enzyme or an acid or base.

Our diet contains a wide variety of proteins from different sources. It is generally accepted that the relative concentration of dietary essential amino acids is the major factor determining the nutritional value of food protein.[7] Proteins derived from animal sources are considered to be nutritionally superior to those from plants because they contain a better balance of the dietary essential amino acids. Of these egg and milk proteins (casein) are frequently used as reference proteins for evaluating protein quality. Proteins derived from meat and poultry muscle are also of very high quality and fish muscle proteins are equally nutritious.[8] Fish muscle contains an excellent amino acid composition and is an excellent source of nutritive and easily digestible proteins.[9,10] However, because fish is extremely perishable and because chemical composition can vary, the utilization of fish as a basic raw food material presents unique food processing problems.[11]

The muscle of different animals is very similar, containing similar protein and similar amino acid profiles. There are slight differences between fish muscle and the muscle of land animals. These are mainly associated with the differences in muscle structure required for swimming and buoyancy. Fish are supported by a mass of water, thus the muscle fibers require less structural support than those in land animals. Because of this, fish muscle tends to have less connective tissue than muscles from terrestrial animals, resulting in more tender texture. Also, because of the unique movement of fish, the structural arrangement of muscle fibers is quite different from terrestrial animals. A large fraction of commercially utilized fish stocks are cold adapted or poikilothermic, and because of this their muscle proteins have different biochemical properties compared with those of endothermic animals.[12] Poikilothermic characteristics of fish proteins make them more heat sensitive than mammalian muscle proteins, with a greater tendency to denature at elevated temperatures.

Fish muscle proteins from cold water species are more prone to denaturation than those from tropical waters.[13,14] The T-50 values (temperature required for 50% denaturation) of fish muscle proteins are also influenced by pH and were reported to be 29 to 35°C at pH 7.0 and 11 to 27°C at pH 5.5.[15]

Protein composition in muscles varies by muscle type. Of the three types (striated, smooth, and cardiac muscle) of muscles, the striated muscles are the predominant form in fish. Striated muscle tissue is arranged into muscle fibers that are bound together by a connective tissue to make a fiber bundle. Fish muscle has "white" and "dark" meat.[15] The white meat is generally more abundant, contains less lipids than the dark meat, and is the most widely consumed type of muscle tissue. It is composed of about 18 to 23% of protein, depending on the species and time of harvesting. Fish proteins can be divided further into different groups based on their solubility. About 70 to 80% of fish muscle are structural proteins. These structural protein are soluble in cold neutral salt solutions of fairly high ionic strength. The remaining 20 to 30% contain sarcoplasmic proteins that are soluble in water and dilute buffers, and a final part of the structural protein, 2 to 3%, being insoluble connective tissue proteins.[11] Recent studies, however, challenge these generally accepted solubility data, showing that the muscle protein components can be highly soluble at low ionic strenghts.[16,17]

Myofibrillar proteins are the primary food proteins of fish, comprising 66 to 77% of the total protein in fish meat. The myofibril protein complexes contain myosin and actin. These are the main components of the thick filament, and thin filament, respectively. Myosin comprises 50 to 60% of the myofibrillar contractile proteins, and actin only 15 to 30%.[12,15] Myosin is the most abundant of the single muscle proteins, making up around 38% of the total, and is a large molecule containing two identical heavy chains (223 kDa) and two light chains (22 and 18 kDa). The molecule has two identical globular head regions that incorporate the light chains and a significant fraction of the heavy chains. The tails of the heavy chains form very long α-helices that wrap around each other[18] (Figure 1). Myosin can be cleaved by proteases at two sites on the molecule, one recognized by both trypsin and chymotrypsin and the other by papain. Papain cleaves near the head region, releasing the head from the tail. Trypsin and chymotrypsin cleave further from the head, dividing the molecule into two components called the heavy meromyosin (with the head region) and the light meromyosin, both with different functional properties.

Myosin molecules are connected via their head region to the polymerized actin molecules in the thin filaments due to the ATPase activity of the head molecules. This complex is called actomyosin and is responsible for muscle contraction and relaxation. Actomyosin plays a major role in determining the quality of fish meat because it is quite labile and easily affected during processing and storage. For example, during frozen storage the actomyosin becomes progressively less soluble and the flesh becomes increasingly tougher.[19]

The thin filament is a complex of actin molecules making a double helix. Tropomyosin sits within the grooves of the thin filaments and two

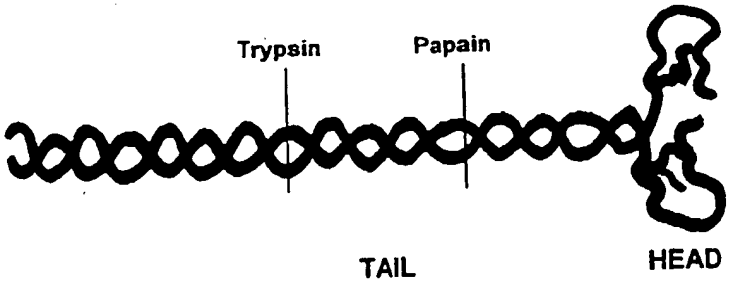

FIGURE 1. Fish myosine molecule.

troponin molecules bind the actin filament at each helical repeat. Actin is the most prominent protein of the three protein in the thin filaments, making up about 13% of the total muscle proteins. Actin occurs in two forms, G-actin, a spherical monomer, and F-actin, a large polymer that connects to myosin. The thin filaments play a very important role by regulating muscle contraction. From the point of view of muscle biochemistry, thin filaments are very important, however, their content is low in meat and their role with respect to food processing has not been studied completely. Other contractile proteins of interest are C-protein, α-, and β-actinin, connectin and paramyosin; however, they are of limited interest as food proteins. With respect to protein hydrolysis, the myofibrillar protein myosin, actin, or actomyosin are subject to enzymic cleavage and are the greatest focus here.

III. PROTEIN HYDROLYSIS

Proteolytic modification of food proteins to improve palatability and storage stability of the available protein resources is an ancient technology.[20] Hydrolysates can be defined as proteins that are chemically or enzymatically broken down into peptides of varying sizes.[21] Protein hydrolysates are produced for a wide variety of uses in the food industry, including milk replacers, protein supplements, stabilizers in beverages and flavor enhancers in confectionery products. The benefits of hydrolyzing food proteins to make functional protein ingredients and nutritional supplements is a more recent technology, with the first commercially available protein hydrolysates appearing only around the late 1940s. Although production is massive worldwide, the proper control of the process and the exact mechanism behind protein hydrolysis is in most cases not fully understood. Recent advances have given researchers insight into the connection between the process/extent of hydrolysis and the physicochemical mechanisms responsible for specific functional properties of the hydrolyzed protein. Recent research on enzyme catalysis has also aided with the proper selection of enzyme catalysts and processing conditions to obtain better control over the reaction and characteristics of the final product.

Chemical and biological methods are the most widely used for protein hydrolysis with chemical hydrolysis used more commonly in industrial practices. Biological processes using added enzymes are employed more frequently, and enzyme hydrolysis holds the most promise for the future because it results in products of high functionality and nutritive value. The chemical and biological hydrolysis are discussed in more detail below, with an emphasis on hydrolysis with added enzymes. In addition, there are many potential techniques for extracting protein from animal tissue. These include the use of aqueous and organic solvents; the conventional processes of cooking, pressing, drying, and hot oil extraction.[22] The extraction of protein by means of solvent is also worth mentioning due to its industrial and historical importance for fish protein recovery.

A. Chemical Methods for Protein Hydrolysis

1. Chemical Extraction: The Making of Fish Protein Concentrate

The extraction methods mentioned above, other than the chemical and biological hydrolysis methods, do not hydrolyze protein. They are used primarily to concentrate intact protein by the removal of water and oil from the substrate. The method of solvent extraction has been frequently employed when producing fish protein concentrate (FPC). The development of FPC was one of the earliest attempts to recover fish protein from processing waste and to produce a protein ingredient from underutilized species. FPC was the precursor to the field of enzyme hydrolysis of fish proteins. A small but extensive research program on the large-scale production of FPC by the Bureau of Commercial Fisheries, now the National Marine Fisheries Service (NMFS) of the Department of the Commerce, began in 1961. The general aim of the program was to study the manufacture and use of FPC as a solution to global protein malnutrition and as a potential economic stimulus to the American fishing industry.[23] Solvent-ex-

tracted FPC (type A FPC) is produced by using primarily isopropanol or azeotropic extraction with ethylene dichloride, although other solvents such as ethanol have been used successfully as well. A standard process presented by Sikorski and Naczk[24] shown in Figure 2 is to grind a whole or eviscerated fish, extract it with isopropanol at a low temperature (20 to 30°C) for 50 min, then collect the supernatant and extract it twice again, first at 75°C for 90 min in isopropanol and then at 75°C for 70 min with azeotropic isopropanol. The final supernatant fraction is collected, dried, milled, and screened to separate out bone particles. The final product has a high biological value and is colorless and odorless, with less than 1% lipids. The problem with type A FPC is that it is not readily soluble or dispersible in foods and has poor emulsification properties.[9,25,26] Dubrow et al.[27]

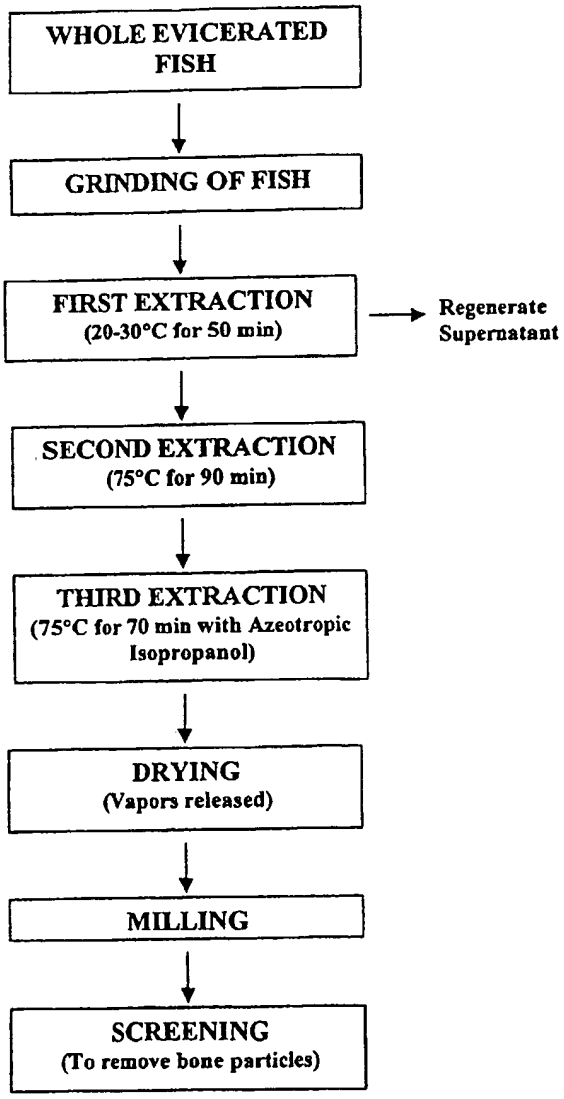

FIGURE 2. A production scheme for fish protein concentrate. (Adapted from Ref. 24.)

reported that FPC produced at higher temperatures (50°C) compared with lower temperatures (20°C) had significantly lower emulsifying properties, but both had very poor solubility. General poor functionality, off-flavors the high cost of production, and traces of solvent in the final product made solvent extracted FPC commercially unsuccessful despite concerted efforts.[3,28] Although FPC lacks solubility, it reportedly has good foaming properties over a wide range of pH (pH 2 to 11), making strong, stable foams.[29-31] Despite problems with protein functionality, solvent extraction is the method of choice for the abundant fatty pelagic fish species such as sardine, herring, and capelin because the protein is effectively separated from the lipids, thereby reducing stability problems normally associated with residual oxidizable lipid. For fatty fish, isopropanol was a slightly more efficient solvent than ethanol considering the residual amounts of lipids, but absolute ethanol produced FPC of lighter color and a neutral flavor.[32]

Many studies with FPC have also been conducted with solvent-extracted FPC as a substrate for enzyme hydrolysis, both to defat the substrate and to make it more accessible to enzymatic hydrolysis, with excellent functional and nutritional properties.[5,25,33,34,35,36] However, enzymatic hydrolysis using FPC as a starting substrate resulted in loss of some functional properties because of excessive protein breakdown but increased nitrogen solubility.[30] Taste and odor problems are generally minimized with a FPC starting material.[33]

Recent studies with solvent-extracted FPC have produced FPC with better protein functionality. For example, recently Vareltzis et al.[37] studied the addition of ethanol-extracted FPC made from sardine to hamburger patties and found that the overall functional properties (water binding and cooking yield) and the penetration depth and shear force value of the hamburger increased with the addition of FPC. However the hamburgers had a slightly unfavorable fishy flavor. Hoyle and Merritt[5] found that herring protein extracted with ethanol in a similar manner and then hydrolyzed with either Alcalase or papain produced a hydrolysate with a markedly reduced bitterness and less fishy odor.

2. The Chemical Hydrolysis Process

Chemical hydrolysis of proteins is achieved by cleaving peptide bonds with either acid or base. Several processes have been proposed for the acid or alkaline hydrolysis of fish.[33] This has been the method of choice in the past for the industry primarily because it is relatively inexpensive and quite simple to conduct. There are however, many limitations to food ingredients using this method. Chemical hydrolysis tends to be a difficult process to control and almost invariably leads to products with variable chemical composition and functional properties.[21,38] Protein hydrolysis with strong chemicals and solvents is performed at extreme temperatures and pH and generally yield products with reduced nutritional qualities, poor functionality, and restricted to use as flavor enhancers.[39,40]

a. Acid Hydrolysis

Acid hydrolysis of proteins is used more commonly than hydrolysis under alkaline conditions. A vast majority of hydrolyzed proteins consumed in the U.S. are prepared by acid hydrolysis, mostly from inexpensive vegetable protein sources that otherwise would have poor nutritive and little functional value in foods. Although the process is harsh and hard to control, it is still the preferred method for hydrolyzed vegetable proteins. Hydrolyzed vegetable protein, which are widely used for flavor and taste enhancement properties, require extensive acid hydrolysis.[38] Applications of hydrolyzed vegetable proteins are primarily as flavoring agents in processed meat, crackers, and soup mixes. Acid hydrolysis of fish protein has usually involved reacting fish proteins with hydrochloric acid, or in some cases sulfuric acid and the proteins are completely hydrolyzed at high temperature, and often high pressure. The hydrolysate is then neutralized to pH 6.0 to 7.0 and concentrated to either a paste or further dried.[4] Because the product is hydrolyzed extensively its primary functional property is high solubility. Total hydrolysis of fish protein substrate can be achieved in 18 h at 118°C in 6N hydrochloric acid.[42] In addition, following the neutralization of

the digest, the hydrolysate contains large amount of salt (NaCl), which can make the product unpalatable and interferes with functionality in food systems. Another drawback of acid hydrolysis is the destruction of tryptophan, which is an essential amino acid. Orlova et al.[43] proposed an acid hydrolysis process of whole fish, where steam distillation is used to remove aromatic substances followed by filtration then concentration. The concentrate was used in dehydrated soup cubes and as a microbial media.[43] The acid hydrolysis is also widely utilized to convert underutilized and secondary raw material from fish into fertilizer due to the low production cost and resulting extensive hydrolysis.

b. Alkali Hydrolysis

The use of alkali reactants, primarily sodium hydroxide, to hydrolyze protein often results in poor functionality and more importantly can adversely affect the nutritive value of the hydrolysate. Despite this, limited alkali treatment is used in the food industry to recover and solubilize a broad range of proteins. For example, mechanically deboned turkey residue (MDTR) includes a significant proportion of alkali-soluble proteins that can be recovered by alkali treatment and used in food applications. Fonkwe and Singh[44] discussed the use of alkali extraction to recover MDTR with an alkaline sodium chloride solution but found it to be unsuitable due to low recovery. Alkaline hydrolysis of fish proteins has primarily used FPC as the starting substrate. During alkaline hydrolysis of fish protein, rapid cleavage to large water-soluble polypeptides takes place, followed by further degradation at a slower rate. Alkali treatment can aid in modifying the properties of insoluble FPC.[24] Tannenbaum et al.[45,46] have studied the alkaline process for hydrolyzing insoluble FPC and its applications. They developed a small-scale batch process that utilizes high pH (12.5) and 95°C for 20 min. The product consisted of large peptides, some relatively insoluble at the isoelectric point, but with an overall improvement in functionality with respect to the original FPC. Use of the solubilized FPC as a milk substitute gave a product far superior to that obtained with FPC starting material, which had poor solubility and dispersibility.

Several deleterious reactions occur in alkaline solutions during hydrolysis. These are initiated by hydrogen abstraction from the alpha carbon of an amino acid and include racemization of L-amino acids, which produces D-amino acids, which are not absorbed by humans. Also, disulfide bonds are split with loss of cysteine, serine, and threonine via β-elimination reactions and formations of lysinoalanine, ornithinoalanine, lanthionine, and β-amino alanine can also occur.[31] Some of these elimination and addition reactions may lead to the formation of toxic substances (e.g., lysinoalanine) that are highly undesirable in foods.[47,48] Alkaline hydrolysis reaction products have an inhibiting effect on proteolytic enzymes, reducing the rate of hydrolysis.[49] Some of the possible reaction products that may form during alkali hydrolysis are shown in Figure 3.[24] High collagen solubility is also observed with alkali treatment.[50]

B. Biochemical Methods for Fish Protein Hydrolysis

Biochemical hydrolysis to produce fish or other food protein hydrolysates is performed by utilizing enzymes to hydrolyze peptide bonds. This can be done via proteolytic enzymes already present in the fish viscera and muscle (endogenous proteases), or by adding enzymes from other sources. To understand the process of enzymatic hydrolysis, it is very important to understand the nature and activity of proteolytic enzymes.

1. Proteolytic Enzymes

Enzymes are biochemical catalysts vital for living beings, because they accelerate chemical reactions between organic constituents within the cells that otherwise would take an extremely long time to complete. In food science and technology enzymes are exploited to perform desired functions in processing and analysis and to facilitate the conversions of raw materials into higher-quality, more desirable foodstuffs.[51] Enzymes make this possible because the active site of an enzyme

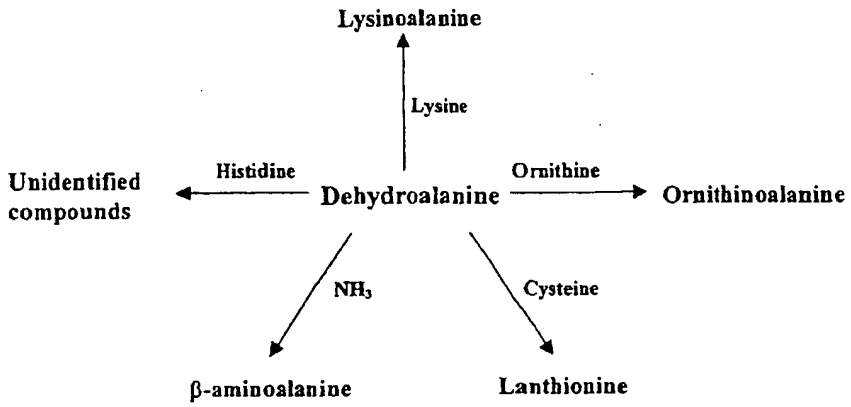

FIGURE 3. Possible chemicals that may form in the alkali-treated proteins.

is highly specific for certain substrates. Enzymes catalyze only one specific reaction and function by forming a complex with the substrate whose transformation they catalyze.

Enzymes used by the food industry and in food research are predominantly hydrolases, most of which are carbohydrases, followed by proteases and lipases. Proteases are among the best characterized enzymes. Proteolytic enzyme preparations are economically the most important group of enzymes, and their use is well established in the food industry.[52] Proteases are categorized according to the specificity of the peptide bonds they attack (hydrolyze) and the mechanism by which they act.[48] Four major classes of proteases are known. They are designated by the principal functional group in their active site: serine, thiol, carboxyl and metallo.[53] Proteases are characterized further by their hydrolyzing mechanism into endoproteinases or exopeptidases. The endoproteinases cleave/hydrolyze the peptide bonds within protein molecules, usually at specific residues to produce relatively large peptides. The exopeptidases systematically remove amino acids from either the N terminus, called aminopeptidases, or the C terminus, called carboxypeptidases, by hydrolyzing the terminal peptide bonds. In food protein hydrolysis, endoproteinases are always used, but occasionally endoproteinases are combined with exopeptidases to achieve a more complete degradation.[20]

Although the four classes of proteases mentioned above have different catalytic mechanisms, they all share a common transition state (intermediate) during catalysis. To discuss enzyme kinetics in any detail would require a separate review, and this is not intended here. However, it is important to know the basic steps in enzyme catalysis and to understand the mechanism of protein hydrolysis. Some proteases preferentially catalyze the hydrolysis of bonds adjacent to a particular amino acid residue, while others are less specific. The catalysis by proteases occurs primarily as three consecutive reactions: (1) the formation of the Michaelis complex between the original peptide chain and the enzyme, (2) cleavage of the peptide bond to liberate one of the two peptides, and (3) a nucleophilic attack on the remains of the complex to split off the other peptide and to reconstitute the free enzyme.[20,54] The hydrolysis of peptide bonds leads to an increase in the numbers of ionizable groups (NH_3^+ and COO^-), with a concomitant increase in hydrophobicity and net charge, a decrease in molecular size of the polypeptide chain, and an alteration of the molecular structure leading to the exposure of the buried hydrophobic interior to the aqueous environment.[55-57] Giving the substrate the symbol S, the enzyme E and the peptides in the reaction P, the overall mechanism can be presented as:

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES \xrightarrow{k_2} EP + H - P'$$

$$\xrightarrow[H_2O]{k_3} E + OH - P + H - P'$$

This enzyme-substrate complex may dissociate back to reactant substrate and free enzyme, or to free enzyme and product molecules. In other words, classic Michaelis-Menten kinetics apply.[20] The generally accepted mechanism for proteases indicates that the second step is the rate-determining step, thus k_2 primarily determines the overall reaction speed, and K_m is more or less equal to the true dissociation constant. This simple mechanism does not, however, deal with the detailed question of how the enzyme and substrate are bound or what molecular configurations lead to product formation. To fully understand the catalysis, a fairly detailed explanation is in order, which is not the purpose of this review.

Enzymatic hydrolysis of proteins is a complex process because of several peptide bonds and their specific accessibility to enzymatic reactions.[47] The specificity of enzymes is not the only factor that affects the peptide profile of the final product. Environmental factors such as temperature and pH play an important role. Both temperature and pH can greatly affect the enzyme reaction kinetics, and the effect of these factors is different for each enzyme. Generally, there is an optimum combination of both pH and T, where an enzyme is the most active. Temperature and pH extremes deactivate the enzymes by denaturing them.

2. Autolytic Hydrolysis

Biochemical production of fish protein hydrolysates may be carried out by employing an autolytic process. An autolytic process depends on the action of the digestive enzymes of the fish itself. There are no enzyme costs involved, and it is a simple operation.[59] The end product of autolytic hydrolysis is generally a fairly viscous liquid rich in free amino acids and small peptides. The digestive enzymes in question are primarily the serine proteases trypsin and chymotrypsin, and the thiol protease pepsin, all major enzymes of fish viscera and digestive tract. Lysosomal proteases, or catheptic enzymes, present in fish muscle cells also contribute to proteolytic breakdown to some extent.

The endogenous enzymes in autolytic hydrolysis are a very complex mixture of enzymes, all with different activity requirements, which result in end products of different molecular profiles. Another complication is that the presence of certain digestive enzymes and their concentration may be highly seasonal, gender and age specific, and can vary tremendously within a species as well as between species. These variations make it very hard to control the hydrolytic process, and direct the production of hydrolysates with specific molecular properties. Autolytic methods such as chemical methods often result in a final product with bad functionality. Despite these problems, endogenous proteolytic enzymes are used to produce hydrolyzed products, specifically fish sauces and fish silage.

The production of fish sauce preceded fish silage production and is the major fermented fish product presently consumed in the world. Its production has thousands of years of tradition in Asia, and it is also known to have been produced in Mediterranean countries in ancient times. Presently, fish sauce is used mainly as a condiment on rice dishes like the popular Nuoc-Nam produced in Vietnam, and the annual production in Southeast Asia is about 250,000 metric tons.[60] The production of fish sauce does not require elaborate processing equipment. The substrate is usually fish from one or more pelagic species, such as anchovies or sardines, or minced whole fish of low commercial value. The substrate is immersed in a solution containing high concentrations of salt (20 to 40%) and at relatively high temperatures, preferably ambient tropical temperatures. In the case of whole fish, the visceral proteolytic enzymes start by hydrolyzing the stomach contents, then work their way through the stomach wall, and finally reach the muscle tissue, where they join with catheptic enzymes to hydrolyze fish muscle proteins. The pH of the fish sauce process is usually neutral, because no base or acid is added to the reaction mixture. The acid-dependent proteolytic enzymes, such as pepsin and the catheptic enzyme, contribute little to fish sauce production. The endogenous serine proteases very slowly breakdown the fish muscle for 6 to 12 months (Nuoc-Mam) under anaerobic conditions. This slow but extensive breakdown results in a liquefied fish sauce composed predominantly of free amino acids, with up to 50% nitrogen recov-

ery. The high concentration of salt is responsible for the slow hydrolysis because this slows the activity of the proteolytic enzymes. More importantly, the high salt concentration and anaerobiosis totally inhibits growth of spoilage microorganisms once the salt has fully penetrated the tissue.[61] Lower concentrations of salt, however, result in sauces with higher yield, lower levels of volatile acids, and better balanced composition of amino acids.[62] During autolysis to produce fish sauce, a lipid phase, an aqueous soluble phase that contains much protein but little lipid, and insoluble sediment of protein and lipid is formed.[63] After the hydrolysis is completed, the liquid protein hydrolysate is tapped, filtered, and bottled, with the final product containing up to 10% free amino acids and low-molecular-weight peptides, and 25% salt.[26,60] Although the production of fish sauce does not improve the nutritive value of the protein, the keeping quality is greatly increased and organoleptic characteristics are generally improved.[64]

The process of manufacturing fish silage is different, but the product shares many characteristics with fish sauce. The production of fish silage was not started until the middle of this century, and it is far from being as widely employed as fish sauce production. The application of fish silage is primarily for animal feed production instead of food applications. The process is rapid and enzymes involved are very effective in producing oil and proteins fractions that are readily separated. The substrate is usually secondary raw material from fish processing or underutilized fish species. The substrate is mixed with strong mineral acids or organic acids such as formic acid to acidify the mixture below pH 4. At this pH the serine proteases are generally inactive, but pepsin and the catheptic enzymes are highly active. The pepsin content can be very high in the visceral portion of fish, and the enzyme is primarily responsible for fish silage production. Under acidic conditions the active endogenous enzymes partially hydrolyze the fish over several weeks to produce a slurry containing up to 12% amino acids and peptides. Usually about 80% of the protein in acid fish silage become solubilized after 1 week at temperatures around 23 to 30°C.[65] The rate is primarily dependent on conditions such as ambient temperature and relative amounts of the visceral organs present. The processing time is much shorter than for fish sauce because no salt is added.[26,60] The production of fish silage using lactic acid bacteria as the hydrolyzing agent has been reported.[66,67] The bacterial fermentation is initiated by mixing minced or chopped fish with a fermentable sugar that favors the growth of lactic acid bacteria, which is advantageous because the bacteria produces acid and antimicrobial factors that inhibit competing bacteria.[65]

The feed applications of fish silage are primarily limited to young animals due to the extensive hydrolysis of the proteins. For fish silage to be incorporated successfully in animal feeds, it has to contain the majority of the nitrogen fraction as intact proteins or peptides rather than as free amino acids, which are less well absorbed. A shorter processing time and added commercial proteases may be useful in such instances. Also, problems connected with the development of bitterness in the hydrolyzed silage can make the product highly unpalatable not only for humans but also animals fed feeds rich in fish silage. The utilization of fish silage as a primary protein source in fish feeds has been neither fully investigated nor commercially successful; however, it is incorporated into diets for pigs, poultry, and mink.[67]

Research on fish hydrolysates made with endogenous enzymes for human food applications has been very limited. Fish sauce is almost the only autolytically produced food of aquatic origin. The main reason for this is fairly straightforward. To produce a functional protein hydrolysate with specific properties, a good knowledge of the enzymes involved is crucial. Endogenous enzymes in fish are a complex and highly variable mixture, and thus the properties of functional protein hydrolysates so prepared may vary greatly under the same reaction conditions. Also, in the U.S. the sale of processed fish foods containing visceral material of any kind is prohibited by the FDA. Despite this, some work on developing fish silage for human consumption has been conducted. In 1972, Malcolm B. Hale and a group of scientists at NMFS and the University of Maryland conducted a comprehensive study on making functional FPH by enzymatic hydrolysis for human consumption.[33] This study involved autolysis of

red hake (*Urophycis chuss*), a relatively lean fish, and alewife (*Alosa pseudoharengus*), a very fatty fish. The autolysis of raw hake was conducted at optimal conditions for the native enzymes, 50°C and pH 7.0, for 24 h where fish was 50% of the total slurry. The resulting hydrolysate was either spray dried or tested as a concentrate. The average yield of dry solids for hake was lower than for any other proteolytic enzyme employed, 10.0% (dry solubles/wet fish), compared with the high of 14.3% from using Alcalase. Similarly, the chemical score attained for soluble hydrolysate were quite low due to low tryptophan content that is correlated with a low recovery. The protein efficiency ratios for both red hake and alewife hydrolysates were essentially equivalent to that of casein. Also, the inclusion of insoluble solids in the final product resulted in very high fat content. The results for lipid pressed alewife were similar. However, by lowering the reaction period to 4 h and raising the temperature to 55°C, a satisfactory product with good nutritional value was prepared. Both the red hake and alewife were only 50 to 70% soluble, requiring substantial additions of commercial enzymes, at uneconomic levels, to become fully soluble. The alewife hydrolysate also suffered from very fishy taste; however, the red hake hydrolysate had a less fishy taste and odor. Food applications of the products obtained by Hale and co-workers were limited and could be used primarily as a protein supplement in cultures where its taste would be acceptable and the caloric value of the lipid desirable.[33]

Shahidi et al.[59] hydrolyzed ground capelin (*Mallotus villosus*) by endogenous enzymes and found that it enhanced the overall extraction of the fish protein at both acid and alkaline pH, as both acid and alkaline proteases are present in fish muscle and viscera. The protein recovery of hydrolysates produced autolytically was, however, considerably lower compared with commercial enzymes, 22.9% compared with 70.6% with Alcalase. A recent study by Cui[68] with chum salmon (*Oncorhynchus keta*) mince and visceral content showed a surprisingly extensive and rapid hydrolysis at an acid pH and 37°C. The hydrolysate also showed a marked difference in the molecular weight distribution of peptides when compared with a commercial pepsin hydrolysis. The native acid enzymes resulted in a product with the majority of peptides of lower molecular weight than the pepsin hydrolysate under the same experimental conditions. This indicates that protein hydrolysates can be obtained through autolysis very efficiently at relatively mild temperatures. The functional properties of the product were, however, not investigated.

The main limitation of work performed on autolytic hydrolysis of food proteins is the lack of research on functional properties. Studies have shown that protein recovery can be adequate and that nutritional requirements are good, but information on functional properties of the resulting hydrolysate is very important to successfully evaluate its use in formulated foods.

3. Enzymatic Hydrolysis of Fish Muscle Proteins with Added Enzymes

Using added enzymes to hydrolyze food proteins is a process of considerable importance used to improve or modify the physicochemical, functional, and sensory properties of the native protein without jeopardizing its nutritive value, and often protein absorption is improved. These enzyme-based processes occur under mild conditions over a series of stages and do not produce hydrolytic degradation products via racemization reactions observed with both acid and alkaline hydrolysis.[69] The process of using added enzymes instead of chemicals or endogenous enzymes offers many advantages because it allows good control of the hydrolysis and thereby the properties of the resulting products.[59] Processes can be designed to take advantage of substrate specificity and the relative reaction rates of different enzymes under the reaction conditions employed. The physicochemical and functional properties of hydrolyzed fish proteins are discussed separately in a later section.

Enzymatic hydrolysis has been employed on a variety of different proteins derived from livestock and poultry meat,[39,44,47,70,71] milk,[57,72–76] and plants.[77,78] Hydrolysis of fish and other aquatic foods is also being seen more frequently in the literature. Several different aquatic protein sources have been investigated for the production of func-

tional fish protein hydrolysates. These include *Rastelliger canagurta* and *Barbus carnaticus* both Indian tropical fishes,[79,80] hake (*Urophycis chuss*),[10,25,33] shark (*Isurus oxyrinchus*),[81,82] sardine (*Sardina pilchardus*),[35,36,83,84] herring (*Clupus harengus*),[5] crayfish,[85] lobster (*Panulirus* spp.),[86] pollack (*Theragra chalcogramma*),[87] capelin (*Mallotus villosus*),[59] dogfish (*Squalus acanthias*),[88] chum salmon (*Oncorhynchus keta*),[68] Pacific whiting (*Merluccius productus*),[89] and Atlantic salmon (*Salmo salar*).[90] It can readily be seen from the list above that the majority of the sources represent underutilized species or are connected to utilization of processing wastes.

Enzymatic hydrolysis of fish protein has been employed primarily as an alternative approach for converting underutilized fish biomass, which is commonly used in making feed or even fertilizer into edible protein products.[15,88] More recently, fish processing waste, or, more appropriately, secondary raw material, has been connected to FPH studies. In many cases this is due to strict government waste regulations. Many processors are no longer allowed to discard their offal directly to sea, resulting in a very high cost of refining the material before discarding. Secondary raw material is the material remaining after fillets are removed, and if viscera is included, this can represent something on the order of 64% of the weight of whitefish, the protein content of this waste being about 10%.[3] Hydrolysis of fish protein with selected proteolytic enzymes provides the possibility of controlling cleavage degree of protein in the substrate. Using suitable enzyme/substrate ratios and reaction times, this permits the production of hydrolysates with different molecular structures and different functional properties that could find applications in various food formulations.[82]

The hydrolytic process and reaction conditions differ between different substrates and enzymes used and also depend on the properties desired for the hydrolysate. Most of the described processes are conducted under research conditions and may have limited applications in the industry. Commercial production of fish protein hydrolysates is still limited on a worldwide basis, but has reached a significant level in a few countries, including France, Japan, and Southeast Asia.[60] Commercial batch protein hydrolysis has several disadvantages such as (1) high cost of using large quantities of enzymes, (2) difficulty in controlling the extent of reaction that can result in nonhomogenous products consisting of fractions of varying molecular weight, (3) low yields, and (4) the need to inactivate enzymes by pH or heat treatment at the end of the reaction, which adds to the processing costs.[91] Also, the enzymes employed in the process cannot be reused.[20] Figure 4 outlines a fairly typical process for producing fish protein hydrolysates. Each step is given a detailed discussion below.

a. The Substrate and Its Preparation

Lean species, or material derived from them, is the substrate of choice for enzymatic hydrolysis as problems with lipid oxidation can be reduced. From an economic standpoint, however, the abundant underutilized pelagic fish would be preferred. The small pelagics comprise 23% of the world's catch,[92] of which only 42% is used as human food. These are mostly fatty species such as herring, sardines, anchovies, and mackerel, and FPH prepared from them would contain high amounts of lipid, which would require additional treatments such as centrifugation to remove excess fat.[93] The fewer steps that are involved in the production, the more economically viable the operation becomes. If a whole fish is used, it is eviscerated and washed, then ground in a meat grinder, usually mixed with an equal amount of water and homogenized in a blender until a viscous homologous mixture is achieved. In some instances a buffer solution is added to the minced fish, for example, phosphate buffer[1,88] and boric acid-NaOH buffer.[85] The presence of buffer salts may affect the final properties of the hydrolysates. In a study of whey protein hydrolysis, Kuehler and Stine[72] decided not to buffer the solutions because of the influence buffer salts might have on foaming or emulsifying properties.

Processes for fatty and lean species are different. If the FPH contains more than 1% fish fat, the fat must either be removed by solvent extraction or stabilized by antioxidants[3,60] such as butylated hydroxytoluene, butylated hydroxyanisol,[5] or propylgallate.[10] A fish protein hydrolysate with high lipid content may darken. The formation of

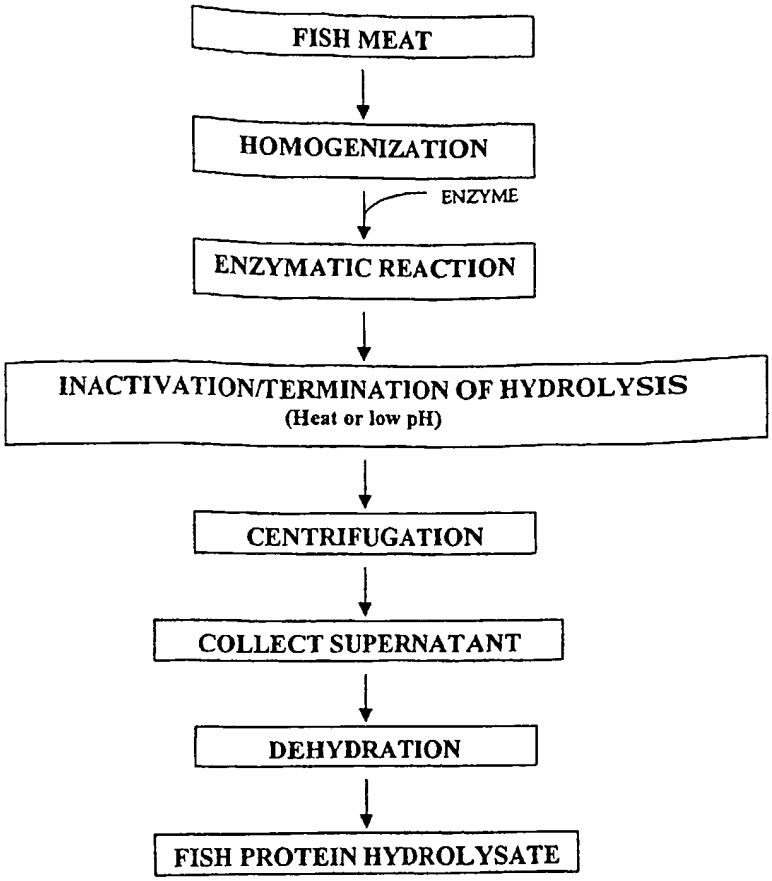

FIGURE 4. A flow sheet for the enzymatic hydrolysis of fish protein to make fish protein hydrolysate

brown pigments may result from aldol condensation of carbonyls produced from lipid oxidation after reaction with basic groups in proteins.[5] Researchers have developed many means of minimizing the lipid content in FPH. To obtain a product of a lipid content not exceeding 0.5% by weight, as established by the Protein Advisory Groups of FAO for a fish protein hydrolysate suitable for human consumption,[94] Quaglia and Orban[36,84] defatted ground sardines by extraction with isopropanol three times (solvent: substrate ratio 1:1) at 46°C for 30 min, and then homogenized the mixture with water. Hoyle and Merritt[5] used an ethanol (90%) extraction directly on minced herring at the fish/ethanol ratio of 1:2 at 70°C for 30 min, then mixed with equal volume of water, hydrolyzed the mixture, then spray dried it. Through this procedure the lipid content was reduced to 0.9 from 4.0% of raw herring. Also before placing the treated substrate in the reaction vessel, chemical agents such as NaCl, sorbic acid, or ethanol are occasionally added to the minced fish to minimize bacterial degradation,[24] especially if reaction conditions are at neutral or alkaline pH. However, adding NaCl can reduce the rate of hydrolysis, increasing reaction time. Ethanol can also adversely affect the reaction process in too high concentration by inhibiting protease activity, although sorbic acid has not been found to affect hydrolysis in concentrations up to 0.5%.[24]

b. The Choice of Enzyme

The water mince mixture is added to a reaction vessel where the hydrolysis takes place. Of-

ten a flask, ranging from 0.5 to 3 L with a close-fitting multisocket lid, has been used. The sockets in the lid usually carry: a stirrer, driven by a overhead variable speed motor to ensure adequate mixing of the system, a thermometer to monitor temperature, a pH electrode to monitor pH, and a "pH-stat" device, where acid or base is added to maintain a constant pH (Figure 5). The temperature of the reaction vessel is controlled. After the required temperature is achieved, the pH of the slurry is adjusted to the desired value. It is important that the mixture is well mixed and consistently stirred when the pH is added to allow for uniform distribution of the added acid or base. Processing temperature and pH is normally selected to optimize the kinetics of the selected enzyme or enzyme mixture.[48] A commercial protease is added in varying concentrations depending on the rate of hydrolysis needed. Given a particular enzyme and a particular substrate, any hydrolysis process involves at least five independent variables. These are S (protein substrate concentration: %N × 6.25), E/S (enzyme-substrate ratio in % or in activity units per kg N × 6.25), pH, T (temperature), and t (time).[95]

A wide variety of commercial enzymes exist that have been used successfully to hydrolyze fish and other food proteins. Proteolytic enzymes from plants and microorganisms are most suitable to prepare fish protein hydrolysates.[26] Enzymes used to hydrolyze fish protein have at least one common characteristic: they have to be food grade, and, if they are of microbial origin, the producing organism has to be non-pathogenic.[96] The choice of enzyme(s) is usually determined by a combination of efficacy and economics.[48]

The screening for a suitable enzyme in a process or experiment is very important if the product is to have predetermined properties. The screening process can be conducted in a variety of ways, and there is no standard methodology for this selection, leaving it primarily up to the individual researcher what method is most appropriate. Good examples of selection procedures are found in studies by Hale,[97] Cheftel et al.,[25] Arzu et al.,[98] Rebeca et al.,[1] Baek and Cadwallader,[85] and Kristinsson and Rasco.[99] In the comprehensive study by Hale,[97] the relative activities of more than 20 commercially available proteolytic enzymes were measured for the hydrolysis of a washed and freeze-dried fish protein substrate from haddock. Preliminary tests at 1 h, 40°C, and pH 7 resulted in the plant enzyme ficin to be most active, but with papain, also a plant enzyme, having a much higher relative ranking. When the enzymes were tested at 24 h the picture changed, 60% digestion (the set limit) was achieved fastest by Pronase, which exhibited the greatest activity per unit weight. The enzymes pepsin, papain, and pancreatin were most suitable if the lowest cost per unit of proteolytic activity was to be followed. These cost estimates are less valid today due to the commercial availability of bacterial enzymes.

Although many would prefer to use acid proteases so microbial growth could be more easily limited, they usually yield a product with low protein yield and too excessive hydrolysis for food use.[39,84,86] Therefore, milder enzymes at neu-

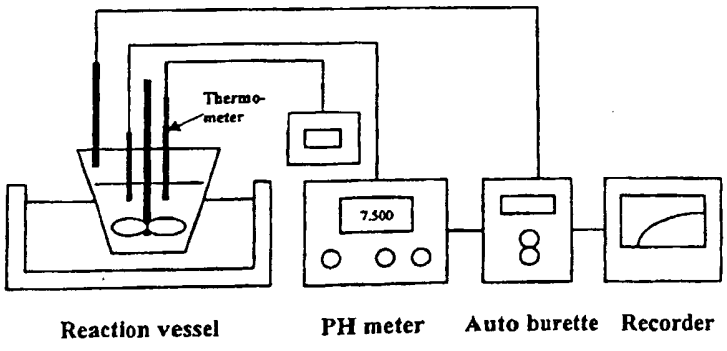

FIGURE 5. A typical enzymatic hydrolysis reaction system in the laboratory. (Adapted from Ref. 20.)

tral and slightly alkaline conditions have been used more frequently in recent years. In some cases, a high level of solubilization is desired. The acid enzyme pepsin has been most successful in solubilizing fish protein. Liu and Pigott[100] produced a high-quality, fluffy, water-soluble fish protein hydrolysate by pepsin hydrolysis of rockfish fillets. Tarky et al.[101] used pepsin at 37°C and pH 2.0 to hydrolyze the entire fish waste resulting from filleted English sole. The final product, after ultrafiltration and spray drying, was a creamy white, nonhygroscopic, water-soluble hydrolysate with low lipid content but very poor nutritional value.

Alcalase, an alkaline enzyme produced from *Bacillus licheniformis* and developed by Novo Nordisk (Bagsvaerd, Denmark) for the detergent industry, has been proven repeatedly by many researchers to be one of the best enzyme used to prepare functional FPH and other protein hydrolysates.[20,36,59,84,88,89] Shahidi et al.[59] successfully used Alcalase to optimize processing conditions to produce capelin protein hydrolysates. Alcalase-treated hydrolysates exhibited superior protein recovery (70.6%) compared with the alkaline protease Neutrase and papain. Alcalase-treated hydrolysates also had the lowest lipid content (0.18%) and excellent functional properties. Quaglia and Orban[36] studied the same three enzymes at optimal conditions on enzymatic solubilization of sardine proteins. Hydrolysates produced using Alcalase and papain were almost identical in nitrogen recovery, which increased with increasing enzyme concentration (70% recovery at a enzyme/substrate ratio of 4%). Neutrase-treated hydrolysates at the same ratio had only over 20% nitrogen recovery. Hydrolysates from Alcalase and papain also exhibited better functional properties and high nutritional value than those from Neutrase. Improved nitrogen recovery of fish protein hydrolysates with increase of protease concentration has been reported elsewhere.[1,79] Alcalase and Neutrase were studied further recently by Benjakul and Morrissey[89] on Pacific whiting solid waste at pH 9.5, 60°C and pH 7.0, 55°C, respectively. Alcalase had a considerably higher activity than Neutrase and led to a more efficient hydrolysis. Optimum conditions for Alcalase were 20 Anson Units (AU)/kg, 1 h reaction time, and waste:buffer ratio of 1:1 (w/v) at 60°C and pH 9.5. The resulting hydrolysate had a high protein content with excellent nitrogen yield (up to 70%) and an amino acid composition comparable to fish muscle. Further, Alcalase was found to be the most cost-effective enzyme out of five enzyme preparations tested to hydrolyzed salmon muscle proteins.[99] Other new enzyme preparations have shown excellent potential for hydrolyzing fish proteins to make highly functional FPH, including Flavourzyme 1000L (Novo Nordisk, Bagsvaerd, Denmark), Corolase 7089 (Rohm Enzymes; Somerset, NJ) and Corolase PN-L (Rohm Enzymes, Somerset, NJ).[99]

In an extensive paper,[33] Hale reported the effects of various processing conditions and commercially available proteolytic enzymes on yield and composition of water-soluble fish protein hydrolysates. He concluded that the hydrolysis of raw hake (*Urophycis chuss*) with Alcalase at pH 8.5 or above gave the best balance of essential amino acids and a high yield of soluble product, followed by pancreatin (a mixture of serine proteases). One of the first studies on added enzyme hydrolysis of fish protein was with papain, due to its favorable properties of pH and temperature optima for activity.[79,80] Two fish species were used as substrate, one freshwater, *Barbus carnaticus*, and the other marine, *Rastrelliger canagurta*, and studied at 40 and 55°C, and pH 5 and 7. Total solids and nitrogen recovery for both species was high, with pH 7 having the highest total solids and nitrogen recovery (69.7% at 55°C for freshwater species) compared with pH 5, possibly attributed to better hydration at pH 7.

To properly compare enzyme activity on the same substrate, it is necessary to determine the general proteolytic activity units at specific reaction conditions. Unfortunately, very few researchers have done this, and most compare enzyme activity on a weight basis of enzymes used in the reaction mixture. Adding enzymes on the basis of weight is meaningless if relative enzyme activity is to be compared, because enzyme activity per weight is different for each enzyme under experimental conditions used. However, there exist few studies that use and compare enzymes on the basis of their proteolytic activity. Gonzalez-Tello et al.[69,102] studied three proteases on whey protein

substrate by adding them to the system based on Anson Units (AU). Unfortunately, they did not use the three enzymes at the same AU. In addition, the activity units were obtained from the manufacturer and not assayed by the researchers. Also, reaction conditions used in the hydrolysis experiments were different from the conditions used in the enzyme assays, thus making the activity units very unreliable.

Benjakul and Morrissey[89] studied the hydrolysis of Neutrase and Alcalase on Pacific whiting solid wastes by adding the enzymes to the system based on AU units. This study has the same limitation as the studies by Gonzalez-Tello et al.[69,102] by using reaction conditions different than those used to assay the enzyme for proteolytic activity and relying on enzyme units provided by the supplier instead of assaying the enzymes themselves. Other studies on fish protein hydrolysis add enzymes according to AU units and also suffer from these same limitations.[59,82] A study by Beddows and Ardeshir[103] is the most carefully conducted study in the literature with respect to using standardized relative enzyme activity. They assayed three proteases, bromelain, ficin, and papain, by using BApNA (Benzoyl-Arg-para-Nitroanilide) to obtain some indication of the relative proteolytic activities of these enzymes. They then added the enzymes to a system of minced Ikanbilis (*Stolephorus* sp.), a tropical fish, at the same activity units as based on their assay. The assay, however, involved different reaction conditions than the hydrolysis experiment, which limited its reliability. Assaying enzymes with BApNA also only estimates the trypsin activity of the enzyme preparation, not their general proteolytic activity. Using BapNA, therefore, gives a less accurate and and probably underestimated value of enzyme activity. In a research conducted by Kristinsson and Rasco,[99] where salmon muscle proteins were hydrolyzed, the enzymes were assayed under the same reaction conditions as they were used in the hydrolysis experiment. A synthetic protein substrate for proteolytic activity, Azocoll, was used to obtain a uniform level of proteolytic activity for all enzymes used. Azocoll is an insoluble cowhide preparation consisting largely of collagen. The method is based on dye release from the insoluble substrate Azocoll when a proteolytic enzyme cleaves the peptide linkages in it. The rate at which the dye is released can be used to quantitatively measure the amount of proteolytic enzyme(s) activity working in a solution by measuring the absorbance at 520 nm. The enzymes were then used to hydrolyze the substrate, all at the same activity unit according to the Azocoll assay, thus comparing them at the same activity level on the same substrate. No reports in the literature have taken this approach; however, the use of Azocoll in fish protein hydrolysis experiments has been reported by Ferreira and Hultin.[104]

c. The Mechanism of Enzymatic Hydrolysis

The enzymatic hydrolysis of fish muscle proteins is characterized by an initial rapid phase, during which a large number of peptide bonds are hydrolyzed, after this rate of enzymatic hydrolysis decreases and reaches a stationary phase where no apparent hydrolysis takes place[59] (Figure 6). The shape of the hydrolysis curve has been associated with enzyme inactivation, product inhibition by hydrolysis products formed at high degrees of hydrolysis, a low K_m value for the soluble peptides that act as effective substrate competitors to the unhydrolyzed fish protein,[1] and possibly autodigestion of the enzyme.[105] Shahidi et al.[59] found that a high concentration of soluble fish peptides in the reaction mixture, released during the initial phase of hydrolysis, reduced both the rate of hydrolysis and the recovery of soluble proteins. Thus, removal of hydrolysate from the reaction mixture should enhance the hydrolysis rate and the protein recovery.

By increasing the protease concentration, and thereby increasing the extent of hydrolysis, recovery of soluble nitrogen increases,[1,71,103] although increasing enzyme concentrations may not be cost effective. Substrate concentration has also negative effects on protein recovery. Linder et al.[47] found that more than 8% protein concentration in the system, regardless of enzyme concentration, seemed to have an inhibiting effect on protein recovery. Baek and Cadwallader[85] reported using Optimase to hydrolyze crayfish processing byproducts the %DH increased as substrate con-

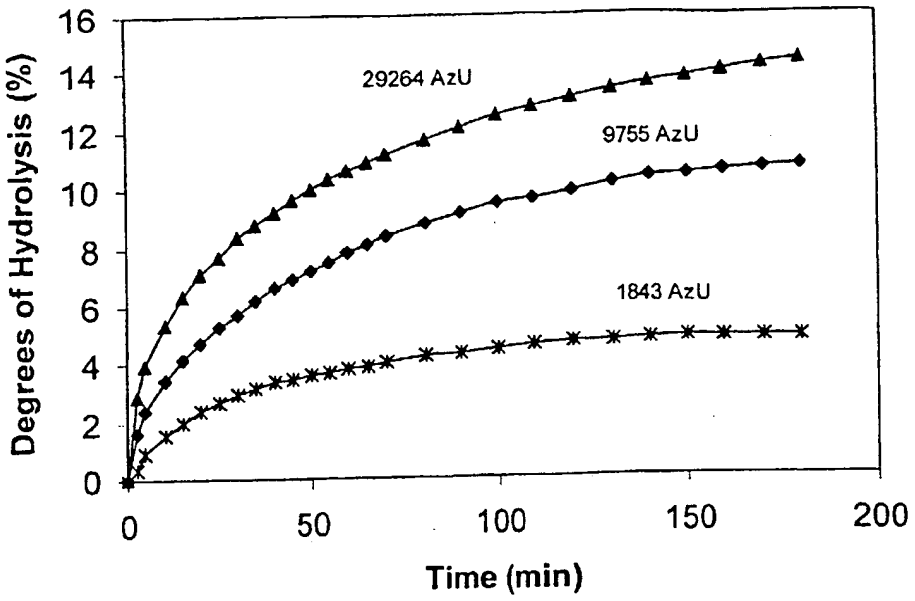

FIGURE 6. Hydrolysis curve for salmon muscle mince with Corolase 7089 at three different activity units (AzU = Azocoll Units).

centration decreased to 45% (w/v), suggesting that high %DH did not coincide with a high amount of hydrolysate. Similarly, Surowka and Fik,[70] who measured the production of protein hydrolysate with Neutrase from chicken heads, reported that hydrolysis increased as substrate concentration decreased. Ferreira and Hultin,[104] using Newlase A to hydrolyze cod (*Gadus morhua*) frames, found that enzyme autolysis can be reduced at higher substrate concentrations.

Because fish tissue is a very complex substrate and also contains large amounts of proteinase inhibitors, it is impossible to explain the mechanisms of protein hydrolysis in detail for this system.[60] A kinetic study of the process is also quite complicated due to the various types of peptide bonds involved and their differing vulnerability to attack by enzymes during the hydrolytic process.[69] Very few studies on kinetics of fish and food protein enzymatic hydrolysis are reported. Sakai et al.[87] conducted a kinetic study on the hydrolysis of pollack surimi protein using a acid protease derived from *Aspergillus niger*, determining the effects of temperature, pH, initial substrate concentration, and enzyme concentration on the kinetics of protein solubilization. Their experimental data followed Michaelis-Menten type kinetics. Michaelis-Menten kinetics has also been observed with whey protein hydrolysis.[69] Kristinsson and Rasco[99] studied the kinetics of five different enzyme preparations during the hydrolysis of salmon muscle proteins and found that the initial rate of the reaction for all enzymes showed a linear relationship to enzyme activity. These experiments indicated that the initial rate constants for each enzyme tested were in the same order. This confirmed previous studies by Hevia et al.[106] and Cheftel et al.[25] on menhaden fish protein as a substrate.

A kinetic model of whey protein hydrolysis with Alcalase has been proposed, where the hydrolytic reaction is zero-order for substrate. The enzyme denatures simultaneously via a second-order reaction due to free enzymes attacking the enzyme bound to the substrate.[69]

Moreno and Cuadrado[107] hydrolyzed vegetable proteins with Alcalase and found reaction mechanism consistent with substrate inhibition and a second-order deactivation with respect to the enzyme concentration. Enzyme autolysis was de-

pendent on the substrate concentration. Cheftel et al.[25] used Pronase to hydrolyze fish protein concentrate and found that the rate constant decreased with time, and that proteolysis did not follow first-order kinetics with respect to concentration of the peptide bonds. Cheftel et al.[25] suspected this to be due to the multitude of possible substrates in FPC, the number of different proteolytic enzymes present, the inhibitory effects of substrate or self-digestion, as well as the different specificities that are known to be present in Pronase. Archer et al.[108] also studied the kinetics of the enzymatic hydrolysis of FPC. Their research found that the enzyme is initially adsorbed to the surface of the protein, with the initial rate of reaction being proportional to the surface area of substrate exposed to the aqueous phase. The overall kinetics were described by a sequence of two first-order processes, an initial, fast reaction in which loosely bound polypeptide chains are cleaved from an insoluble protein particle, and a second, slower reaction in which a more compacted core protein is digested.[108] Langmyhr[109] studied the kinetics of the hydrolytic breakdown of cod muscle with acetyltrypsin. The results indicated that enzyme molecules are rapidly and firmly bound to fish proteins and that the maximum binding occurred under the optimum pH and T conditions for the enzyme. Results also suggested that muscle proteins are hydrolyzed in the same way and to the same extent whether they occur separately or are integrated into intact muscle.

d. Measuring the Extent of Enzymatic Hydrolysis

To follow the reaction kinetics and get a measure for the extent of the hydrolytic degradation, a parameter named degree of hydrolysis (%DH) is employed. In principle, there are several control methods, but under practical industrial conditions there are few.[96] The degree of the hydrolysis is most commonly used to describe hydrolysis of food proteins. The advantage of using %DH as a process parameter is that %DH appears to determine unambiguously the properties of a protein hydrolysate for a given protein-enzyme system.[96] The degree of hydrolysis demonstrates both theoretically and empirically that four processing variables, S, E/S, T, and t, can be left uncontrolled, provided that %DH is controlled.[95] From this, it is obvious that %DH is a very simple and rapid method of measuring the extent of protein breakdown.

Two methods for measuring %DH have been studied thoroughly and shown to be satisfactory, the pH-stat technique and osmometer technique. The pH-stat method is more commonly used and more useful for industrial applications. The principle behind the pH-stat method is relatively simple and is based on maintaining a constant pH during the reaction. By pH-stat, the %DH is calculated from the volume and molarity of base or acid used to maintain a constant pH. The degree of hydrolysis is defined as the percent ratio of the numbers of peptide bonds broken (h) to the total numbers of bonds per unit weight (h_{tot}; meq/kg protein, calculated from the amino acid composition of the substrate): $\%DH = (h/h_{tot}) \times 100$.
%DH can also be expanded to:

$$\%DH = \frac{B \cdot N_B}{\alpha \cdot h_{tot} \cdot MP} \cdot 100$$

where B = base consumption in ml (or acid in case of acid proteases), N_B = normality of the base (or acid), α = average degree of dissociation of the –NH groups or COOH groups, MP = mass of protein in grams (%N × 6.25). The degree of dissociation is found by the following equation:

$$\alpha = \frac{10^{pH-pK}}{1+10^{pH-pK}}$$

This equation for %DH is valid when hydrolysis is conducted at a pH above the pK of the α-NH group (for hydrolysis at neutral or alkaline pH). Under these conditions, the reaction will result in a net release of protons (H^+) when peptide bonds are cleaved and the base consumption is proportional to the number of peptide bonds split.[110] Above pH 6.5, the dissociation of the protonated α-NH group becomes significant. Therefore, it is

important to know the pK values of the α-NH groups, both to determine the reaction pH and to calculate the dissociation constant correctly. The pK value varies significantly with temperature. The pK values at different temperatures can be calculated according to Steinhardt and Beychok:[111]

$$pK = 7.8 + \frac{298 - T}{298 \cdot T} \cdot 2400$$

where T = temperature in Kelvin.

If samples are drawn during the experiment, it is crucial to correct for the actual value of base consumed. Actual base consumption (B′) will be smaller than the theoretical base consumption (B) if this is the procedure. To correct for this, B can be calculated from the following formula:[20]

$$B = B_1' + \left[\frac{B_2' - B_1'}{\left(\frac{M}{M-m}\right)}\right] + \left[\frac{B_3' - B_2'}{\left(\frac{M}{M-2m}\right)}\right] + \cdots\cdots + \left[\frac{B' - B_{n1}'}{\left(\frac{M}{M-(n \cdot m)}\right)}\right]$$

where B_1', B_2', etc. are the actual base consumption at the drawing of samples number 1, 2, etc. The value n is the sample number and m is the sample size (ml). The pH-stat method, however, has some limitations. Use of pH-stat as a means of control is only practical outside the approximate pH interval 3 to 7.[96] This has to be taken into consideration when deciding on pH values for the hydrolysis reaction. Within this pH range, process control may depend on other methods. Therefore, the pH-stat method for %DH determination is useful mainly under alkaline conditions.

The other commonly used method to measure hydrolysis, the osmometer technique, is more universally applicable. This technique measures the freezing point of samples drawn at regular intervals during hydrolysis to construct a hydrolysis curve. For this method, the freezing point depression (ΔT) is measured and converted to milli-osmol (ΔC) as follows:[95]

$$\Delta T = K_f \cdot \omega \cdot \Delta C$$

where K_f is 1.86 K/mol for water, ω the osmotic coefficient, and C the osmolality of solution. From the increase in osmolality, ΔC, %DH can be calculated according to the following formula:

$$\%DH = \left(\frac{\Delta C}{S\% \cdot f_{osm}} \cdot \frac{1}{w} \cdot \frac{1}{h_{tot}}\right) \cdot 100$$

where ΔC = depression of freezing point measured in milli-osmol, S% × f_{osm} = g protein/1000 ml H_2O, 1/w = calibration factor for the osmometer: 1.04, h_{tot} = total number of peptide bonds in the substrate.

The f_{osm} factor is calculated by knowing the percent substrate dry matter, D%, present in the reaction mixture:

$$f_{osm} = \frac{1000}{100 - D\%}$$

Although the osmometer technique is a very good method to determine the degree of hydrolysis, it is being less employed frequently. The degree of hydrolysis is now being more commonly determined by using either the trichloroacetic acid (TCA) method or the trinitrobenzenesulfonic acid (TNBS) method. Both of these methods are very useful when working within the pH 3 to 7 range, where the pH-stat is unusable. Several variations of these methods exist. The most commonly used TCA method is based on determining the approximate degree of hydrolysis (%DH) of protein hydrolysates by the ratio of percent 10% TCA soluble nitrogen in the hydrolysate compared to total amount of protein in sample. This is generally done by removing aliquots at selected intervals and mixing with 20% TCA to create 10% TCA-soluble and TCA-insoluble fractions. These mixtures are then centrifuged and the supernatant analyzed for nitrogen. The degree of hydrolysis is thus calculated from the following equation:

%DH = (10% TCA-soluble N in sample/Total N in sample) × 100

This method has been used successfully in fish[5] and other food protein hydrolysis.[44,70] The TNBS method is based on the concentration of primary amino groups in the hydrolysate. It is a spectrophotometric assay of the chromophore formed by the reaction of TNBS with liberated amino groups (420 nm), at slightly alkaline conditions.[112] The degree of hydrolysis can then be calculated as presented by Baek and Cadwallader,[85] and Benjakul and Morrissey,[89] for crayfish hydrolysis and whiting hydrolysis, respectively:

$$\%DH = \frac{L_t - L_0}{L_{max} - L_0} \cdot 100$$

where L_t = the amount of a specific liberated amino acid at time t, L_0 = the amount of the specific amino acid in original substrate (blank), L_{max} = the maximum amount of the specific amino acid in the substrate obtained after hydrolysis.

New methods and modifications of previous methods for determining the degree of hydrolysis are being developed. In the special case of fish, a simple method for monitoring the enzymatic hydrolysis of fish protein was developed by Ukeda et al.[113] The degree of hydrolysis was monitored by an amino group determination method with gluteraldehyde (GA). The method is based on the consumption of dissolved oxygen during the reaction between gluteraldehyde and the liberated amino groups of the protein substrate. The results by the GA method were in agreement with the TNBS method previously described, with a correlation coefficient of r = 0.992.

e. Termination of Enzymatic Reaction

When a desired %DH is attained, it is necessary to terminate the enzymatic reaction. This is very important as otherwise the enzymes would remain active in the substrate and further hydrolyze the protein and peptides. Deactivation of enzymes is achieved either by chemical or thermal means. Usually the slurry of hydrolysate and enzymes are transferred to a heat bath, where the enzymes are deactivated by exposing them to temperatures ranging from 75 to 100°C for 5 to 30 min, depending on the type of enzyme. For example, papain is very heat tolerant, and has been reported to need at least 90°C for 30 min to be fully inactivated.[5] Terminating the reaction by thermal means is undesirable[114] because of the effects of heat denaturation on the protein that leads to exposure of hydrophobic residues and subsequently protein aggregation. Diniz and Martin[88] suggest that this form of heating can have the advantage of being very effective in the separation of oil from the fish protein substrate, although Webster et al.[39] found that some protein-fat interaction occurred at elevated temperatures that prevented their separation, when using bovine lungs as the protein substrate.

The temperatures at which various proteins denature and unfold vary enormously. Because fish muscle proteins are adapted to function at lower temperatures, they may not be particularly heat stable. Denaturation is usually undesirable because it results in altered physicochemical properties, particularly a loss in protein solubility and functionality.[31] Spinelli et al.[34] are among few researchers that have recognized the adverse effect elevated temperatures may have on fish protein hydrolysates. To both terminate the enzymatic reaction and quantitatively recover the protein fractions, they reacted enzymatically hydrolyzed muscle proteins from rockfish with 5% sodium hexametaphosphate (HP), following slight acidification to form an insoluble protein-phosphate complex. By isoelectric precipitation, they recovered up to 90% of the protein. In addition the complex could be washed free of occluded nonprotein nitrogen components with no loss of protein nitrogen.

Chemical inactivation would be to either lower or raise the pH of the slurry to a point where the enzyme deactivates. Some enzymes are more sensitive to pH changes than they are to temperature changes. Alcalase is a relatively thermostable enzyme, but it is very sensitive to acid pH. Complete inactivation of Alcalase therefore is obtained by lowering the pH to 4.0.[20,59] Neutralizing the slurry to pH 7.0 would inactivate pepsin and most other acid proteases. Extremes of pH can also like elevated temperatures, have detrimental effects on protein and peptides. Many proteins unfold at pH values less than about 5 or greater than 10. Unfolding at such extremes of pH usually occurs because the folded protein (or oligopeptide) has groups buried in nonionized form that ca

ionize only after unfolding.[52] Stabilizing salt-bridges between ionizing groups can also be disrupted by extreme pH values. The termination of the enzymatic reaction thus is a formidable obstacle in any FPH operation. The choice of inactivation method should be carefully made and based up on what the enzyme under study is sensitive to, whether it is heat or pH. In some cases a combination of elevating temperatures and lowering pH have been used.[82]

f. Protein Hydrolysate Concentration

Commonly, the slurry is desludged by centrifugation, which results in several fractions (Figure 7): sludge in the bottom, aqueous layer in the middle, lipid-protein fraction between aqueous layer and sludge, aqueous and oil layers and oil layer on the top.[68] A single centrifugation step can eliminate the vast majority of the lipids present. The oil layer overlying the aqueous layer is then removed and the soluble fraction collected. Because lipid in the final hydrolysate is a major concern for FPH, it is important to remove it. Lipid residues in FPH must be lower than 0.5% to prevent alteration of the lipid fraction during storage.[34] More than one centrifugation step is often required to separate the soluble proteins from the lipids and insoluble solids. The second centrifugation step would be performed only on the soluble fraction. Other separation methods for fish protein hydrolysates have been reported such as suction filtration of the sludge[82] and filtering the slurry by passing it through a 2-mm mesh screen.[86] The removal of colored and odiferous matter has also been reported by treating the first soluble fraction obtained by centrifugation with 1% w/v charcoal at 55°C and 30 min before the next separation step.[59]

In a commercial operation, the final soluble fraction is generally spray dried to convert the hydrolysate to a powdered form, which can be incorporated into food formulations. The insoluble fraction or the sludge precipitated during centrifugation may be used as animal feed. Spray drying of the soluble fraction is one of the most energy consuming and expensive steps in the production of protein hydrolysates. In industrial production a compromise must be made between the hydrolysate yield and the amount of water that must be removed to obtain a dry product.[60] A suspension of equal amounts of water and fish substrate appears to be convenient from an economic point of view. Rebeca et al.[1] performed a study where eviscerated mullet (*Mugil cephalus*) was enzymatically hydrolyzed without the addition of water, to lower the cost of spray drying. Interestingly, the experiment resulted in higher dry matter content, with high protein recovery, of the soluble fraction and the cost of drying was reduced. Generally, in the laboratory, hydroly-

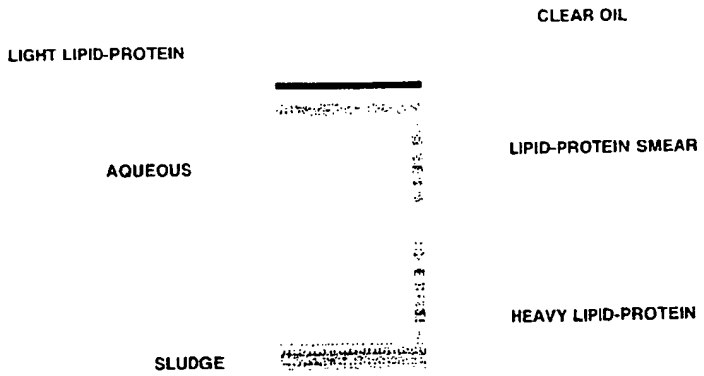

FIGURE 7. Different fractions obtained when recovering soluble fish protein hydroysates.

sates are neutralized and freeze dried. By neutralization the final product can have a fairly high salt content, which is undesirable (HCl + NaOH → H_2O + NaCl). This can be mostly avoided if the soluble fraction is passed through an ion exchange column before freeze-drying. Dialysis to desalt the soluble fraction can also be a useful process.

Increasingly, ultrafiltration membranes have been introduced into the production process for hydrolysates. The hydrolysate after enzyme inactivation is filtered directly through a membrane with a specific molecular weight cut-off value. The ultrafiltration process can contain more than one filter, yielding several molecular weight fractions depending on the product desired. A major advantage for such a process is the control of molecular size of selected peptides so that a uniform product is possible,[76] as the molecular size of the hydrolyzed proteins is a key factor in determining functional properties of hydrolysates.[115] The method has, however, not found its way into fish protein hydrolysate production. The reason probably being that in order to successfully use the ultrafiltration, the sample has to be very pure and free of lipids. It is, however, possible that the method could be applied to highly purified and defatted FPH powders. The method has found to be very useful in soy,[115] whey,[75,116] and casein protein hydrolysis.[76] In the case of whey protein, large-molecular-weight fractions are primarily responsible for allergic reactions. However, small peptides and free amino acids are less well absorbed by humans. Therefore, protein hydrolysis should not be more extensive than the minimum required to eliminate allergic responses. By ultrafiltration therefore it is possible to exclude both the too large and too small peptides and collect the molecular weight in between by passing the hydrolysate solution through two filters, the first with a higher cut-off value than the second.[116] Applying this technique to FPH would be an interesting task to undertake.

IV. PHYSICOCHEMICAL AND FUNCTIONAL PROPERTIES OF FISH PROTEIN HYDROLYSATES

As previously mentioned, one of the major advantages and goals of enzymatically hydrolyzing fish proteins is to modify and improve their functional properties. The functional properties of fish protein hydrolysates are important, particularly if they are used as ingredients in food products.[60] Enzymatic hydrolysis of fish proteins generates a mixture of free amino acids, di-, tri-, and oligopeptides, increases the number of polar groups and the solubility of the hydrolysate, and therefore modifies functional characteristics of the proteins, improving their functional quality and bioavailability. The choice of substrate and proteases employed and the degree to which the protein is hydrolyzed affect the physicochemical properties of the resulting hydrolysates.[105] Enzyme specificity is important to peptide functionality because it strongly influences the molecular size and hydrophobicity of the hydrolysate.[117] Thus, the peptides obtained have different molecular profiles, and the surface energy of the hydrolysate is different, depending on the enzyme used; these variations have a bearing on the functionality of the mixture.[114,118] The more narrow the specificity, the are the larger peptides produced; the broader the specificity, the smaller are the peptides generated. As the range of enzymatic activities within commercial preparations is increased, the hydrolysate becomes more complex.[4] The chain length of peptides or breaking of linkage is also dependent on the extent of hydrolysis; conditions of hydrolysis; concentration of enzyme and the type of protein to be hydrolyzed.[41] Due to the complex peptide profile, it is often useful to calculate the average peptide chain length (PCL) introduced by Adler-Nissen and Olsen[119] to express the composition of the protein hydrolysate. PCL is calculated from the degree of hydrolysis in the following way:

$$PCL = \frac{100}{\%DH}$$

This approximation is acceptable in nearly all practical cases.[20] The relationship between PCL and %DH is shown in Figure 8.

Manipulating the reaction conditions during enzymatic hydrolysis of food proteins produces hydrolysates with different solubility and emulsifying characteristics, foaming properties, or taste characteristics.[4] The control of the enzymatic reaction is very important, as previously discussed. Uncontrolled or prolonged hydrolysis of fish proteins may result in the formation of highly soluble

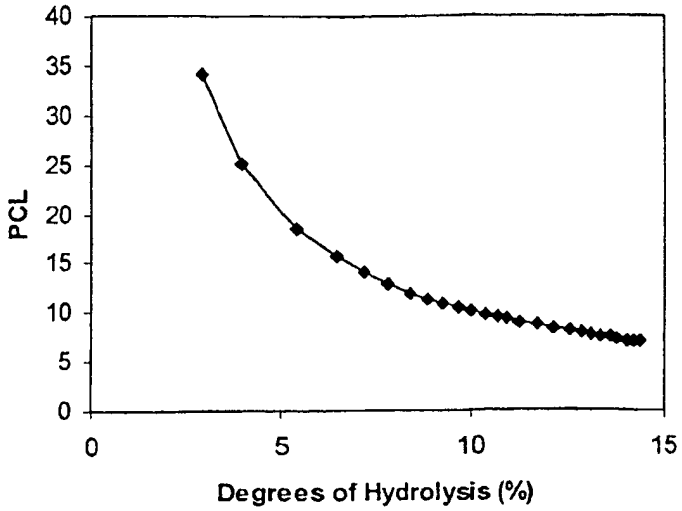

FIGURE 8. The relationship between average peptide chain length (PCL) and degrees of hydrolysis (%DH).

peptides, completely lacking the functional properties of the native proteins. This may promote the formation of undesirable bitter peptides. By controlled hydrolysis it is possible to eliminate these drawbacks and to obtain hydrolysates with different physicochemical and functional properties, in some cases even better than the originals.[36] The physical and chemical properties that govern protein functionality include size, shape, amino acid composition and sequence, net charge and distribution of charges, hydrophobicity/hydrophilicity ratio, peptide structures, molecular flexibility/rigidity, and the ability to interact/react with other components.[120] Functionality of food proteins has been defined as: "those functional and chemical properties which affect the behavior of proteins in food systems during processing, storage, preparation and consumption".[31] The following sections discuss the main functional properties that fish protein hydrolysates exhibit, that is, solubility, water holding, emulsifying, foaming and sensory properties, and research aimed toward evaluating these properties for various FPH preparations.

A. Solubility

Solubility is probably the most important of protein and protein hydrolysate functional properties. Many of the other functional properties, such as emulsification and foaming, are affected by solubility,[121] and therefore it is an excellent indicator of the protein hydrolysate functionality, and its potential (and limitations of) applications.[31,58] Hydrophobic and ionic interactions are the major factors that influence the solubility characteristics of proteins. Hydrophobic interactions promote protein-protein interactions and result in decreased solubility, whereas ionic interactions promote protein-water interactions and result in increased solubility. Ionic residues on the surface of peptides and proteins introduce electrostatic repulsion between protein molecules and repulsion between hydration shells around ionic groups, both major contributors to increased solubility of proteins. Solubility of protein and protein hydrolysates is generally measured by employing the *nitrogen solubility index* (NSI), a standardized method developed by AOCS and later modified by Morr et al.[122] The NSI is determined by suspending a protein hydrolysate sample in water then stirring and centrifuging the mixture. The supernatant is then analyzed for nitrogen content by the Kjeldahl procedure, and the NSI calculated as a percentage of the soluble nitrogen to the percentage of total nitrogen in the sample.[20]

Intact fish myofibrillar proteins have the problem of the lack of solubility in water over a wide range of pH,[13,123] and enzymatic hydrolysis is very important in increasing the solubility of these

proteins. The effects of enzymatic hydrolysis on fish protein are straightforward. Enzymatic breakdown of the protein involves a major structural change in that the protein is gradually cleaved into smaller peptide units, and as the degree of enzymatic hydrolysis increases the solubility of fish proteins increases. The enhanced solubility of the hydrolysates is due to their smaller molecular size compared with the intact protein, and the newly exposed ionizable amino and carboxyl groups of the amino acids, that increase the hydrolysate hydrophilicity.[58,117] To effectively bind to water molecules, the peptides have to have the ability to form hydrogen bonds between its hydrophilic polar amino acid side groups and the water molecules. Hydrolysis exposes some of the hydrophobic groups to the surface, but at the same time converts even more hydrophobic groups to hydrophilic groups by generating two end carbonyl and amino groups. Thus, the smaller peptides from myofibrillar protein hydrolysis have proportionally more polar residues, with the increased ability to form hydrogen bonds with water. This increases protein solubility to that of the intact protein. In addition, ion-dipole interactions between water and simple ions such as Na^+ and Cl^- are also important in the interactions between the polar or charged groups on biomolecules and water.[18] Biomolecules tend to be very soluble at favorable NaCl conditions. The increased solubility is partly through the formation of sodium salts of carboxyl groups of proteins, COONa.[13] This is true for fish myofibrillar proteins, but the hydrophobicity of globular proteins, such as milk proteins, may increase with exposure of apolar amino acid residues after hydrolysis.[117] Although increased solubility has a positive relationship to the extent of hydrolysis, care has to be taken that the substrate is not too extensively hydrolyzed. A very high degree of hydrolysis may lead to high solubility, but this can have very negative effects on the rest of the functional properties. To maintain or improve functionality, generally low degrees of hydrolysis are necessary.[4]

Sugiyama et al.[84] studied enzymatic hydrolysis with several alkaline, neutral, and acid proteases on defatted sardine meal. The result showed that all the alkaline proteases had higher ability to produce highly soluble protein hydrolysates compared with the neutral and acid ones. Gel chromatography showed that hydrolysates prepared with the alkaline proteases had a lower average molecular weight. The increased hydrophilicity of these preparations could be due to their larger charge to size ratios compared with the longer proteins such as those found in nonhydrolyzed proteins from a similar source.[44] Interestingly, pepsin had lower solubilized protein ratio than all of the alkaline proteases, but pepsin is generally considered one of the best enzymes to solubilize fish protein.[35,36,68,97,100] Quaglia and Orban[35,36] also studied the properties of hydrolysates produced from sardine and concluded that Alcalase and papain, at optimum pH and temperature, both gave hydrolysates characterized by high solubility. Hydrolysates made with Alcalase at higher degrees of hydrolysis showed a decrease in high-molecular-weight fractions, and increased solubility.[35] This connection between %DH and solubility is also reported for other food protein.[124] A significant increase in solubility was observed with an increase in %DH from 2.5 to 15% for an enzymatically hydrolyzed single cell microbial protein ("Pruteen"), from less than 10% solubility to over 90%.[124] Similar results were observed with soy protein hydrolysates from 1 to 8.3% DH.[96] Yu and Fazidah[125] hydrolyzed *Aristichthys noblis*, a Chinese freshwater fish, with protease P "Amano" 3, and reported excellent solubility at 15% DH after a 3-h digestion.

Hoyle and Merritt[5] found that enzymatically hydrolyzed ethanol-extracted herring FPH had highest solubility compared with nonextracted enzymatically hydrolyzed FPH. This finding suggests that the lower lipid content of ethanol-extracted herring FPH may have resulted in less competitive water binding of proteins compared with hydrolysates with higher lipid contents. Vieira et al.[86] studied the functional properties of hydrolysates from lobster processing wastes and found these to be highly soluble, pepsin yielding a fraction with higher solubility than papain or a fungal protease. The highest nitrogen solubility index (NSI) for these hydrolysates was found at extremes of pH. The solubilities of all the products were low at pH 5, approximately corresponding to the isoelectric point of protein, at which it precipitates. Shahidi et al.[59] also found that cape-

lin protein hydrolysates (CPH) prepared by different enzymes had different solubility profiles at different pH conditions. However, in contrast to the findings of Vieira et al.,[86] Shahidi et al.[59] found lower solubility at pH 7 to 8, and with close to 100% solubility for Alcalase at pH 5. The lowest solubility value at pH 7 to 8 was, however, 84%, thus CPH had excellent solubility over the range of pH 2 to 11. Hydrolyzed salmon muscle proteins exhibited excellent solubility at 5 to 15% DH, between 92 to 100%, with over 96% solubility in the pH range of 2 to 11.[90]

The solubility of enzymatically hydrolyzed fish protein is generally much higher than for chemically produced fish protein concentrate (FPC). A FPC type-B air-dried fish powder had extremely poor solubility at a wide range of NaCl molarity and pH,[126] and alkaline hydrolysis of red hake exhibited lower than 30% solubility at pH 1 to 7.[45,46]

Pure fish myofibrillar protein are very insoluble close to their isoelectric point; therefore, high solubility of FPH over a wide range of pH is a very useful property for many food applications, including beverage applications. The good solubility and good nutritive value of enzymatically hydrolyzed fish protein hydrolysates also makes them well suited to produce milk replacers for weanling animals.[1,10,124] Presently, this is being done in Japan and France. In Japan, one company is making "bio-fish flour" by enzymatic digestion of sardines that is used in feeds as a milk replacer for calves and piglets.[60,126] Menhaden hydrolysate produced by pancreatine was found to be an excellent milk replacer with high PER value with the process cost half as much as dried skimmed milk.[127] Soluble FPH is an excellent amino acid source for supplementing cereal proteins[10] and to be used in bakery products, soups, and infant formulas.[1] Pasta enriched up to 3.5% of fish protein hydrolysate increased the protein content by 5%, with a dramatic increase in the dietary essential amino acids lysine (37.5%), valine (31%), and threonine (18%).[128] Soluble FPH from extensive hydrolysis is an excellent source of nitrogen for microbial growth. Extensive hydrolysis results in a product of free amino acids and low-molecular-weight peptides, and therefore has found to be very promising for microbial peptone production.[129] In the U.S., the main application of FPH is not for foods but as weanling feed for piglets, and increasingly as a pellet coating for pet food.[60]

B. Water-Holding Capacity

Water-holding capacity refers to the ability of the protein to imbibe water and retain it against gravitational force within a protein matrix, such as protein gels or beef and fish muscle, and it is positively correlated with water-binding capacity.[120] Water-holding capacity of proteins added to muscle tissue is of great importance to the food industry because retaining water in a food system often improves texture. The functional properties of proteins in a food system depend in part on the water-protein interaction, and the final outcome greatly depends on how well the protein binds and holds water in a food system. However, a successful specific application of a functional protein ingredient or additive for enhancing a single specific functional property may not be transferable to other food systems. Fish protein hydrolysates are highly hygroscopic, and this has to be considered when producing them. Proper packaging and low relative humidity of air during processing is an important consideration. The presence of polar groups such as COOH and NH_2 that increase during enzymatic hydrolysis have a substantial effect on the amount of adsorbed water and moisture sorption isotherm for these materials. The recommended maximum water content of FPH for storage is 0.075 g/g at less than 15% RH.[130]

Fish protein hydrolysates have excellent water-holding capacity, and thus useful properties for certain food formulations. Kristinsson[90] made salmon FPH with several enzymes to 5, 10, and 15% DH and added these to minced salmon patties at 1.5%. Water loss after freezing (48 days) and thawing was reduced to 1% compared with 3% for the control. In this study there was no connection observed between %DH and water loss, FPH made using Alcalase had the best water-holding properties in salmon mince patties, but all FPH exhibited better water-holding properties than egg albumin and soy protein concen-

trate. Onodenalore and Shahidi[82] increased cooking yield of comminuted pork by 2.4 to 9.3% by adding 0.5 to 3.0% of a shark protein hydrolysate. Cooking yield increased with increasing addition, and the hydrolysates obtained via an Alcalase-assisted process from the washed myofibrillar shark proteins were more effective than hydrolysates made from untreated shark muscles. Shahidi et al.[59] found similar results with capelin protein hydrolysates (CPH) when added at a 3% level to communited pork, CPH increased cooking yield of approximately 4%. At even lower addition levels there was a large reduction in the amount of drip loss, indicating that CPH has strong water-binding capacity. In addition, CPH at 0.5 to 3.0% level inhibited the formation of TBARS by 17.7 to 60.4%, suggesting that CPH may have antioxidant properties, perhaps due to chelation effects. Hatate et al.[131] also found that sardine myofibril protein hydrolysates exhibited antioxidant activity. More importantly, hydrolysates appeared to act synergistically with several commercial antioxidants. These antioxidant effects are highly dependent on the amino acid composition and molecular size of the hydrolysate peptides.

Fish protein concentrate (FPC) produced from sardine with an ethanol process has also been studied in a meat model system with respect to water holding. FPC was added to a hamburger-type product made from beef at 20, 40, and 60% addition, and increased percentage of FPC in the formulations significantly improved the cooking yields of the products.[37]

C. Emulsifying Properties

The emulsifying properties of FPH are directly connected to their surface properties, or how effectively the hydrolysate lowers the interfacial tension between the hydrophobic and hydrophilic components in food. Proteins adsorb to the surface of freshly formed oil droplets during homogenization and form a protective membrane that prevents droplets from coalescing.[6] Hydrolysates are surface active and promote oil-in-water emulsions because they have hydrophilic and hydrophobic functional groups and are water soluble.[121] Hydrolysates orient their hydrophobic loops in the apolar oil phase, while the polar segments extend into the aqueous phase. Desirable surface active proteins and protein hydrolysates have three major attributes: (1) ability to rapidly absorb to an interface, (2) ability to rapidly unfold and reorient at an interface, and (3) an ability, once at the interface, to interact with the neighboring molecules and form a strong cohesive, viscoelastic film that can withstand thermal and mechanical motions.[120,132]

Emulsifying capacity and emulsifying stability are two methods generally used to measure the ability of protein hydrolysates to form and stabilize emulsions. Emulsifying capacity is usually defined as the volume of oil (ml) that can be emulsified by the protein hydrolysate (g), before phase inversion or collapse of emulsion occurs.[31] Because FPH produces emulsions of low viscosity, the most accurate way to measure this is to use an oil titration method,[133] and measure the electrical resistance during oil titration. A sudden increase in resistance is observed when the maximum emulsifying capacity is reached. Emulsion stability refers to the ability of an emulsion to resist changes in its properties over time[134] and can be determined simply using gravitational methods. Measurement involves blending the hydrolysate with oil and water, centrifuging, and measuring total volume of emulsion, its stability expressed as the difference between total volume of an emulsion and the aqueous volume to total volume.[135,136]

The emulsifying properties of hydrolyzed protein are improved by carefully controlling the extent of hydrolysis. Extensive hydrolysis results in a drastic loss of emulsifying properties.[58] Although small peptides are highly stable and diffuse rapidly and adsorb at the interface, they are less efficient in reducing the interfacial tension because they cannot unfold and reorient at the interface, like proteins with higher molecular weight.[137] Solubility seems to play an important role in emulsification because rapid migration to and adsorption at the interface are critical.[138] However, complete solubility is not an absolute requirement. Solubility and emulsifying properties have been found to correlate up to 25% protein solubility.[120] Casein hydrolyzed with pancreatin showed a linear decrease in emulsifying

activity with increase in %DH. Casein hydrolysate at 67% DH consisted solely of amino acids, di-, and tripeptides with drastically reduced emulsifying activity.[57] Smaller and smaller peptides are formed as the enzymatic hydrolysis progresses and this impacts emulsifying properties.[138] Protease specificity also plays a key role in the emulsifying properties of protein hydrolysates because this strongly influences the molecular size and hydrophobicity of the resulting peptides. Kuhler and Stine[72] found that whey protein hydrolyzed with Prolase yield large-molecular-weight peptides with excellent emulsifying stability and activity. In contrast, Pronase, which has a broad specificity, produced much smaller peptides and yielded hydrolysates with very poor emulsification properties. Hence, a careful choice of enzymes and low degrees of hydrolysis are recommended if good emulsifying properties are desired.

There is a relationship between %DH and emulsifying properties for fish protein hydrolysates. Enzymatic hydrolysis had a negative influence on the capacity to form and stabilize emulsions as degree of hydrolysis increased for sardine protein hydrolysates[83] and salmon protein hydrolysates.[90] Different molecular weight distributions of the hydrolysates show that a higher content of high-molecular-weight protein fractions plays an important role in stabilizing emulsions. Hydrolysates with lower degrees of hydrolysis have higher surface hydrophobicity and sardine hydrolysates at low %DH have better emulsifying capacity than commercial sodium caseinate.[83] Cui[68] obtained similar results for enzymatically hydrolyzed chum salmon muscle.

Hydrophobicity plays an important positive role in determining emulsifying properties. Kato and Nakai[139] reported that effective hydrophobicity determined fluorometrically showed significant correlation with interfacial tension and emulsifying activity of a wide variety of proteins studied. Li-Chan et al.[140] also found that surface hydrophobicity predicted the emulsifying properties of meat proteins. A positive correlation between surface activity and peptide length has been found,[73] and it has since been generally accepted that a peptide should have a minimum length of >20 residues to possess good emulsifying and interfacial properties.[141]

Various emulsification properties have been found for hydrolyzed seafood protein products. Hydrolyzed processing wastes from lobster using papain and an undefined fungal protease had a rather poor capacity for oil emulsification.[86] Also, relatively poor emulsifying capacity and stability were reported for enzymatically produced capelin protein hydrolysate[59] and shark protein hydrolysates;[82] however, neither of these studied defined the %DH of the products. Enzymatically hydrolyzed myofibrillar proteins from rockfish fillets showed an increase in emulsifying capacity and stability with hydrolysis compared to the intact protein.[34,123] Emulsifying capacity reached a point at which there was no change with %DH.[34] Hydrolysates from rockfish muscle made with bromelain had relatively poor emulsifying capacity and yielded unstable emulsions.[136] Removing lipids from rockfish protein hydrolysates resulted in a dramatic loss in emulsifying capacity; the loss increased with increasing extraction temperature.[34]

Fish protein derivatives with good emulsifying properties can also be prepared by acetylation.[60] Acylating agents such as acetic or succinic anhydrides can react with the amino groups of proteins in an alkaline environment, forming amide groups.[24,142] The acetyl residues participate in the formation of additional hydrogen bonds or else can contribute to hydrophobic adherences, thus increasing emulsifying properties. Groninger and Miller[136,142] are among researchers that reported an increase in emulsifying properties of fish protein by acetylation. Miller and Groninger[136] found that emulsifying properties of FPH prepared by bromelain increased until 43 to 59% of the free amino groups were acetylated, with further acetylation having no effect.

Emulsifying properties of fish protein concentrates have also been reported.[27] Fish protein concentrate produced by isopropanol extraction had a decreased emulsifying capacity with increased solvent extraction. This loss of emulsification capacity was believed to be tied to a loss in solubility.

The emulsifying ability of other food proteins, primarily milk protein, has been studied extensively. Enzymatically hydrolyzed whey protein had excellent emulsifying capacity and stability when made with the protease Prolase (~3.0 g

oil/mg protein).[72] Pepsin and Pronase hydrolyzed whey protein yielded emulsions of less capacity and stability, both decreasing with time of hydrolysis and %DH.[72] The degree of hydrolysis was, however, not specified. Each of these enzymes have different substrate specificities and action patterns. Pepsin and Pronase hydrolysates had higher %DH values than Prolase hydrolysates. Enzymatic hydrolysis progressively decreased the emulsifying activity index of casein, with a more than twofold reduction after hydrolysis to a %DH of 58%, with an additional small decrease at 67% DH.[57] A drop in emulsifying capacity was highly correlated with a drop in hydrophobicity, and reduced molecular size, and thus in agreement with our findings.[90] Trypsin hydrolysis was found to greatly improve the emulsifying properties of casein and whey protein and was highly influenced by pH.[138]

Chicken head hydrolysates[70,71] and turkey waste hydrolysates[44] have also been tested for emulsifying capacity, and both had poor emulsification ability. A significant drop in emulsifying properties as hydrolysis progressed was observed when alpha-chymotrypsin was used to hydrolyze peanut protein fractions, reaching a stationary phase after a certain time.[78]

There are many different factors that may account for the difference observed between hydrolysates in both the ability to form an emulsion and emulsion stability. Peptides molecular characteristics and peptide chain length are the major reason for the different emulsification ability of hydrolysates. Environmental conditions such as pH, ionic strength, temperature, etc. also have an effect on the emulsification properties. Also, some synergistic effects of peptides on emulsifying properties have been noted.[117] In short, the foregoing indicates that peptide behavior is complex and not easy to explain.

D. Foaming Properties

The chemistry underlying foaming properties of protein and protein hydrolysates have many things in common with emulsifying properties. Both rely on the surface properties of protein. Food foams consist of air droplets dispersed in and enveloped by a liquid containing a soluble surfactant lowering the surface and interfacia tension of the liquid.[31] The amphiphilic nature o proteins makes this possible; the hydrophobi portion of the protein extends into the air and th hydrophilic portion into the aqueous phase Townsend and Nakai[143] showed that total hydro phobicity, or the hydrophobicity of exposed o unfolded protein, have a significant correlation t foaming formation. The surface hydrophobicity which is important for emulsification, does nc correlate with foam formation.

Many different methods have been develope to measure foaming properties of proteins an protein hydrolysates. Most, if not all, of thes methods have the drawback of poor experiment reproducibility, thus making it hard to compa findings between laboratories. Foaming prope ties are usually expressed as foam formation ar foam stability. Phillips et al.[144] developed a meth where the ability of protein to form foams described as overrun. Overrun is the percenta of excess volume produced by whipping a prote containing liquid compared with the initial vc ume of the liquid. Foam stability is measured b whipping the protein solution and measuring t time required to decrease half of the volur Foam stability is a highly empirical method, b is simple to perform and has found useful app cations with FPH.

Very few studies have been performed foaming properties of fish protein hydrolysat Shahidi et al.[59] and Onodenalore and Shahid studied the foam formation, or whippability, a foam stability of shark and capelin protein h drolysates, respectively. In both processes Alcal was the protease used for hydrolysis. The cape protein hydrolysate had good whippability, 90 at a 12% DH. Shark protein hydrolysates fr shark meat had higher whippability, 106%, a hydrolysates from washed shark myofibrillar p teins had whippability of 130%. Capelin hydr sates had higher foam stability after 30 s t dropped significantly compared with hydroly shark myofibrillar protein. Hydrolyzed whey p tein isolates prepared using Alcalase had ex lent foaming formation and stability at a 3% DH The foaming ability for whey was much hig than for shark and capelin protein hydrolysat

There is a connection between the degre hydrolysis and foaming properties. Kuehler

Stine[72] concluded that whey proteins hydrolyzed to a limited degree had a increased foaming ability but reduced foam stability. This was attributed to more air being incorporated into solution of smaller peptides, but the smaller polypeptides do not have the strength required to give a stable foam. This was seen in the initial 30 min of hydrolysis with further hydrolysis resulting in peptides that lack the ability to stabilize the air cells in the foam. Weak foams are commonly observed when food proteins are hydrolyzed. However, the advantage of using hydrolyzed proteins as foaming agents is their insensitivity to change in pH. The pH of the dispersing medium markedly affects foaming, particularly foam stability, with foaming properties being highest close to the isoelectric point of the protein.[146] Fish protein hydrolysates and especially fish protein concentrates (FPC) have the unusual property of having good foaming properties, and of making strong, stable foams over a wide pH range.[29-31] The foam formation of FPC is provided by the soluble proteins, only 1% of total FPC, while the remaining denatured proteins act as foam stabilizers.[30]

Foaming properties of fish protein hydrolysates can be improved by acylation. Groninger and Miller[147] succinylated hydrolysates produced by hydrolyzing rockfish fillets with bromelain. These foams were significantly more stable compared with foams from modified soy protein and egg white; however, they had smaller foam volume. The succinylated protein foams were more stable over a wide range of pH (3 to 9) compared with the other protein. The succinylated FPH was incorporated into a dessert topping, a soufflé, and both a chilled and a frozen dessert, and the products were as acceptable to a taste panel as similar foods containing no fish protein. Ostrander et al.[148] incorporated succinylated FPH, as prepared by Groninger and Miller,[147] into whipped gelatin desserts. The succinylated FPH formed a stable foam with smooth texture, highly acceptable with respect to flavor and mouthfeel.

E. Fat Absoption

Various methods for the measurement of fat absorption have been described. Commonly, hydrolysates are mixed with a specified amount of excess fat for a particular time and then centrifuged at a low centrifugal force and the fat adsorption expressed as the millilter of fat bound by 1 g protein hydrolysate[59,90] for FPH. The mechanism of fat absorption, as assessed by this method, is attributed mostly to physical entrapment of the oil, and thus the higher bulk density of the protein the more fat absorption.[31] Fat-binding capacity of proteins correlates with surface hydrophobicity.

Fat absorption of salmon protein hydrolysates produced using different enzymes to 5, 10, and 15% DH was studied by Kristinsson.[90] The FPH at all %DH exhibited excellent fat absorption, greater than both egg albumin and soy protein concentrate. The hydrolysates at 5% DH had significantly higher fat absorption (5.98 to 7.07 ml oil/g FPH) than 10% DH (3.22 to5.12 ml oil/g FPH) and 15% DH (2.86 to 3.86 ml oil/g FPH) due to the larger peptide sizes. Only two other publications dealing with fat absorption for FPH could be found, for capelin protein hydrolysates[59] and shark protein hydrolysates.[82] Both studies fail to define the units used to express fat absorption, and thus it is impossible to compare them to other studies.[90]

Other protein hydrolysates have been studied with respect to fat absorption. Casein hydrolysates with excellent oil-holding capacity, made with papain, were dissociated by SDS, indicating that hydrophobic interactions were primarily responsible. The substrate specificity of enzymes also seemed to play a major role in this.[114] This was further confirmed by Kristinsson,[90] who showed that different enzymes used to hydrolyze salmon muscle protein had different fat absorption ability. The capacity of a hydrolysate to absorb fat/oil is an important attribute that not only influences the taste of the product, but it is also an important functional characteristic that is required especially for the meat and confectionery industry. Fish protein hydrolysates therefore could very well be used in such applications.

F. Sensory Properties

Although enzymatic hydrolysis of proteins develops desirable functional properties, it has

the disadvantage of generating bitterness. This is a common problem with fish protein hydrolysates, and the major reason for its slow acceptance as a food ingredient. The mechanism of bitterness is not very clear, but it is widely accepted that hydrophobic amino acids of peptides are a major factor. In fact, the hydrophobicity of peptides can be measured, and it has been found that most bitter peptides have values over 1400 kcal/mol, whereas nonbitter peptides have values below 1300 kcal/mol. This principle is, however, only valid for molecular weights below 6000 D.[149] Peptides with a molecular weight from 1000 to 6000 D and with hydrophobic characteristics have shown most likely to be bitter.[102] Hydrolysis of protein results in exposing buried hydrophobic peptides, which will be readily able to interact with the taste buds resulting in detection of bitter taste. An extensive hydrolysis to free amino acids, however, decreases the bitterness of these bitter peptides because hydrophobic peptides are far more bitter compared with a mixture of free amino acids. Free amino acids, are however, undesirable from a functional standpoint. Strict control of any hydrolysis experiment and termination at low %DH values therefore is desirable to prevent the development of a bitter taste and the retention of functional properties.

Common features found in the structure of bitter peptides are, for example, N, or C, terminal basic amino acid residues, C, or N, terminal hydrophobic peptide fragments and peptide chain folding due to proline residues within the peptide structure.[150] Bitterness is due to particular arrangements of certain chemical groups in the peptides. Two functional groups are necessary to produce bitterness, such as a pair of hydrophobic groups or a hydrophobic or a basic group.[151] The sensation of bitterness may also be exerted by chemical compounds having a hydrophobic region and one hydrophilic group spaced 0.3 nm apart.[95] Because enzymes have different preferences for amino acids, choosing the most appropriate enzyme preparation for hydrolysis can control the bitterness. Enzymes with a high preference for hydrophobic amino acids such as Alcalase are often preferred and frequently yield products of low bitterness.[20,96] The use of exopeptidases, as opposed to endoproteinases, can also be helpful in overcoming the bitterness in fish protein hydrolysates, particularly exopeptidases that split off hydrophobic amino acids from bitter peptides. However, for an enzyme preparation to be effective for hydrolyzing protein, both exopeptidases and endoproteinases are required. Many studies have shown that proteolytic preparations containing exopeptidases and endoproteinases produce less bitter peptides than single proteases.[152-155] Trials have shown that by the additional action of exopeptidases in proteolytic processes, it is possible to move the bitterness point up to higher degree of hydrolysis.[156]

Despite the problem with bitterness of fish protein hydrolysates, little research has been conducted. While FPH is highly nutritious and safe and functional properties are good, the sensory properties are extremely important for the successful adaptation and acceptance by the food industry and the consumer. Yu and Fazidah[125] found a connection between the degree of hydrolysis and the intensity of bitterness when hydrolyzing *Aristichthys nobilis* with a commercial protease. As the %DH increased so did the bitterness, and after a 5-h hydrolysis samples were described as "very bitter". Interestingly, no bitterness was observed until after 4 h of hydrolysis. Papain-hydrolyzed herring had higher bitterness scores than Alcalase-hydrolyzed herring.[5] Alcalase hydrolyzed samples at higher degree of hydrolysis were less bitter than papain-hydrolyzed samples at the same %DH. The greater hydrophobic specificity of Alcalase compared with papain could result in a reduced bitterness. Enzymatically hydrolyzed ethanol-extracted herring, with either Alcalase or papain, had very low bitterness, suggesting that reduced bitterness could be due in part to lower lipid content. Lalasidis et al.[157] hydrolyzed cod filleting offal with Alcalase, which produced a product with a bitter taste. This bitter taste was eliminated when the hydrolysate was treated further with pancreatine, which is rich in exopeptidase activity.

Fish protein hydrolysates have been incorporated into food systems to evaluate their acceptability, and sometimes with satisfactory results.[147,148] Proteins from *Oreochromis mossambicus*, a freshwater fish, were hydrolyzed with Alcalase to produce a soluble hydrolysate that was incorporated into crackers.[158] Sensory

evaluation showed the fried crackers with up to 10% addition of FPH were highly acceptable.

Vieira et al.[86] hydrolyzed lobster processing waste for 5 h with several commercial enzymes and found no difference in taste between the hydrolysates and a control sample, and detected no bitterness. This is perhaps due to the extensive hydrolysis. The hydrolysate possessed a distinct, pleasant smell, and it was proposed for use in various formulated food products such as flavor enhancers. Is has long been known that proteases can be used for recovery of flavor.[159] Baek and Cadwallader[85] attempted to fully utilize crayfish processing byproducts as flavorings by hydrolyzing them with Optimase at optimal conditions. They found that it is possible that the hydrolysates could serve as feedstocks for the production of value-added seafood flavor extracts. Fujimaki et al.[160] reported that a fish protein concentrate treated with Pronase had a flavor potentiating activity like that of monosodium glutamate (MSG) but was accompanied by an unfavorable bitter flavor. A low molecular acidic peptide fraction contributed significantly to this MSG-like flavor. The acidic peptides isolated later from the same fish protein hydrolysate showed that at least four dipeptides (Glu-Asp, Glu-Glu, Glu-Ser and Thr-Ser) and five tripeptides (Asp-Glu-Ser, Glu-Asp-Glu, Glu-Gln-Glu, Glu-Gly-Ser, and Ser-Glu-Glu) had a flavor quantitatively resembling that of MSG, but with weaker intensities.[161] Hevia and Olcott[162] analyzed basic tripeptides that were responsible for bitter taste in fish protein hydrolysates. The tripeptides contained asparagine and lysine as the second and C-terminal residues, respectively, with the N-terminal residue leucine or glycine.

Many techniques have been suggested to reduce or mask bitterness in hydrolysates, but few of them applied to FPH. These include treating hydrolysates with activated carbon that partly removes bitter peptides with absorption,[59,163] extracting bitter peptides with solvents,[163] application of further enzyme treatment with exopeptidases,[152] and by using the so-called plastein reaction.[164] Chakrabarti[165] reported success in debittering fish protein hydrolysate using ethyl alcohol. Higher concentrations of alcohol lowered the bitter fraction in the hydrolysate.

The plastein reaction is among few that have been studied with respect to fish protein hydrolysates. Figure 9[166] outlines the steps involved in the plastein reaction. Heck[164] investigated the plastein reaction in FPH from peptic hydrolysis and found that it reduced the bitterness but with an accompanied loss of FPH solubility. Some success using the plastein reaction has been reported with fish waste[167] and sardines.[168]

Plastein can be defined as a protein substrate that may be prepared by reversing hydrolysis with a protease such as pepsin or papain in a concentrated protein hydrolysate.[60,169] The plasteins are a mixture of peptides of varied composition and molecular weight between 1000 and 500.000 D.[24,166] Three factors are necessary for completion of the plastein reaction: (1) a low-molecular-weight protein hydrolysate as a substrate, (2) a high substrate concentration, and (3) a reaction environment of pH 4 to 6 regardless of the enzyme involved.[164] During plastein synthesis, a high concentration of hydrolysate (30 to 50%) is incubated with an enzyme resulting in a condensation of the peptides. As new polypeptides are formed, they aggregate via hydrophobic associations. The final product has a low lipid content and special hydrophobic functional properties.[31,41,60] Tanimoto et al.[170] have studied the mechanism of plastein synthesis with α-chymotrypsin and found that it proceeds in two stages. The first stage is characterized by a reaction of the peptide with the hydroxyl group of Ser 195 that produces a peptidyl-α-chymotrypsin complex that then undergoes aminolysis as a result of a nucleophilic attack of a second peptide. The plastein reaction has also been found useful for protein recovery from extensively autolytically produced fish silage.[171] It is strongly recommended that this process be studied further for fish protein hydrolysis.

V. FUTURE DEVELOPMENTS AND POTENTIAL APPLICATIONS

The use of FPH as a functional food ingredient still has a long way to go until it becomes economically feasible and accepted by industry and consumers. However, there is a wonderful opportunity for this to happen, due to regulations

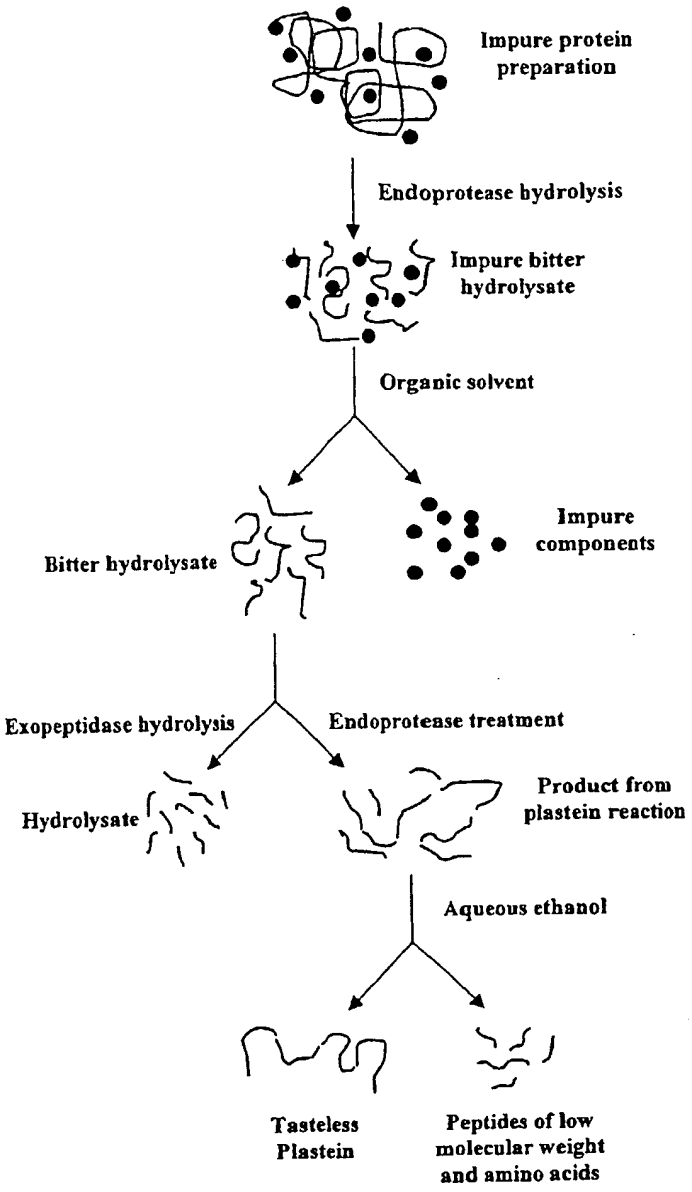

FIGURE 9. The plastein reaction. (Adapted from Ref. 166.)

of processing waste and the abundance of small pelagic underutilized species that will be the frontiers of fisheries in this century. These factors should stimulate the conversion of low value fish materials into more valuable and palatable products. Other applications outside the food industry may also be a feasible option for FPH. Soluble FPH from extensive hydrolysis is an excelle source of nitrogen for maintaining the growth different microorganisms. Previously, it was not that extensive hydrolysis of fish protein results a product containing free amino acids and lo molecular-weight peptides, which is very promi ing for microbial peptone production.[129]

The beneficial chemical composition of FPH and FPC has also led to using these materials as fertilizer with good results. The FPH might be too expensive for this purpose, as added enzymes are used, and the product is usually dried. However, Ferreira and Hultin[104] developed an enzymatic process using the acid protease Newlase A to extensively hydrolyze cod frames. This process is currently being used. The use of FPC as fertilizer is more common, either from chemical hydrolysis or autolysis. A successful process using secondary raw material from processing plants (frames and viscera) and using the acid endogenous enzymes to extensively hydrolyze the material is being operated in Washington State. The final product is sold in bulk to local farmers, mainly cranberry growers, and with superior results compared with other commercially available fertilizers.[172] The mechanism behind the effect of the FPH and FPC to stimulate growth and development better than synthetic fertilizers need to be studied further. Novel applications taking advantage of plant growth-stimulating effect of FPH and FPC could possibly be developed. An extensive research program supervised by Dr. Kalidas Shetty at the University of Massachusetts at Amherst examines the effect of FPH on plants. In one study the effects of using FPH to stimulate somatic embryogenesis in Anise (*Pimpinella anisum*) when compared with proline, a known stimulator, was examined.[173] The conclusion was that FPH could well become a proline and amino acid substitute in plant tissue culture applications. The positive effects of FPH due to proline and its precursor glutamate on plant growth was confirmed in a recent study by the same group, where melon (*Cucumis melo* L.) shoot organogenesis was stimulated.[174] Proline and glutamate can be obtained from FPH and potentially can be used for value-added applications in the plant propagation industry,[174] a new arena of application for FPH and possibly a very valuable one.

Using FPH and FPC for animal feed applications due to its good amino acid balance and high protein content could be quite feasible. As mentioned previously, fish silage is used primarily for this purpose and is essentially limited to young animals, due to the extensive hydrolysis of the proteins.[67]

Another little-studied property of FPH is its antioxidant potential. Studies have found powerful antioxidant activity in intact fish protein[131] and in hydrolysate.[59] These effects are probably highly dependent on the amino acid composition and molecular size of the FPH peptides and deserve further study.

VI. CONCLUSION

There have been several attempts to make functional fish protein hydrolysates with enzymes, some successful and some not. Most of these studies are rather crude, perhaps due to the applied nature of the field, and have many shortcomings, specifically failing to compare enzymes at the same activity level, controlling the %DH properly and characterizing the chemical and functional properties of the final products. There is potential for these products to be produced and sold as functional food ingredients, but at present other applications such as plant nutrients, fertilizers, and animal feeds might be more feasible. Standardized procedures to examine the functional properties are needed, as well as more studies on using endogenous enzymes to make functional FPH. With more basic research on the molecular level the future of FPH could be bright, especially in light of the environmental problems facing fisheries. It is time for FPH research to take a new, fresher, and more scientific direction, because the field has been more or less stagnant for the last 20 years or so.

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